THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXVII | JULY, 1960 | NUMBER 1

REPAIR OF THE OLFACTORY MUCOSA

WITH SPECIAL REFERENCE TO REGENERATION OF OLFACTORY CELLS (SENSORY NEURONS)

EDWIN W. SCHULTZ, M.D.

From the Department of Bacteriology and Experimental Pathology, Stanford University School of Medicine, Stanford, Calif.

Although the normal structure of the epithelium of the olfactory mucosa has been known for many years, little has been reported on its repair. There are at least two reasons why the possible mechanism of repair of this structure should be of interest: (1) It is a surface structure in which nerve cell bodies ("olfactory cells") or a primitive type of neuron lies in a peripheral tissue. (2) The mucosa in which these cell bodies lie is not separated from the lamina propria by a basement membrane, but rests directly upon a structure of some complexity, from which different elements could participate in the repair.

The present studies are an outgrowth of early work on chemical prophylaxis of experimental poliomyelitis in monkeys.[1,2] They were prompted by observations showing that the resistance afforded by an intranasal irrigation with zinc sulfate solution a day or two prior to intranasal inoculation with a given neuronotropic strain of poliomyelitis virus (MV strain) was usually followed in 3 or 4 months by resusceptibility to intranasal inoculation. The initial resistance induced could be explained by extensive destruction of the olfactory epithelium, thus interrupting the nervous pathway by which this strain could reach the central nervous system. It was not possible, however, for us to explain the ultimate return of susceptibility except on the possibility that some measure of restoration of the nervous pathway occurred in time. These observations, therefore, directed our attention to the possible mechanism

Aided in part by early grants from the National Foundation for Infantile Paralysis, Inc., New York.

Received for publication, September 28, 1959.

underlying the return of susceptibility, and particularly to the possibility that this might be related to a restoration of olfactory sensory neurons (olfactory cells). A preliminary paper on our observations was published in 1941,[1] and enlarged on to some extent in 1942.[2] Further observations have been made from time to time since then, and it is the purpose of the present paper to summarize these.

Gross and Microscopic Structure of the Olfactory Region

The gross anatomy of the nasal passages in the rhesus monkey has been described by Geist.[3] Certain qualifications of his description are required, however, on the basis of our own observations. He stated that in the rhesus monkey, "there is nothing comparable to the human superior concha, this part of the wall being smooth and not appreciably elevated." He therefore recognized in the gross only the two lower conchas, which he designated superior and inferior. While it is true that there is little if any bony ledge in evidence where the superior concha should be, there is a clearly defined, tongue-like fold of soft tissue demonstrable on low power microscopic examination of sections made through the coronal plane. This tongue-like fold begins 4 or 5 mm. below the top of the nasal vault and is 1 or 2 mm. in length. The olfactory mucosa extends from the top of the nasal vault around the fold and down the lateral wall to a little below the level of the end of the fold. This is near the upper surface of what is designated by Geist as the superior concha, but which actually corresponds to the usual location of the middle concha, the upper portion of which in man also marks the lowermost limit of the olfactory mucosa. On the medial side, the olfactory mucosa extends down to about the same level as on the lateral side. Most of the mucosa lines a cleft which in the monkey measures about 1 cm. in depth, 1.5 cm. in length, and 1 mm. in width. The narrow width of the space covered by most of the mucosa is probably a factor of importance in the effect produced by chemical agents, since, in addition to its inherent susceptibility to a given chemical agent, such a narrow cleftlike space would tend to retain the agent and prolong its action.

Information on the microscopic structure of the olfactory mucosa can be found in any histology textbook. The illustrations, however, are likely to be in the form of schematic sketches, and not photomicrographs, as presented in this paper. The literature prior to about 1925 relating to the structure of the olfactory mucosa has been reviewed by Schaeffer,[4] Parker[5] and Hopkins.[6] Little relating to its normal structure has been added since. An extensive bibliography on the anatomy, physiology, and pathology of the olfactory system in vertebrates and invertebrates has recently been published by Airkem, Inc.[7]

Material and Methods

All of the observations were made on monkeys. One per cent solutions of zinc sulfate (C.P. or U.S.P.) in distilled water were employed for the intranasal treatments. These were applied by irrigation of the nasal passages while the animals were under ether anesthesia and suspended in a fully inverted position in a specially designed rack. The irrigations were carried out with a 50 cc. Luer syringe to which a short (12 cm.) rubber tube was attached. The latter carried a small bulb at its terminal end which was fitted snugly into the external nares. The fluid was put through the nasal passage gently while the animal's mouth was held open and the tongue pulled forward with forceps to avoid inhalation of the solution. After one nasal passage had been irrigated, the other was similarly treated. The animals were kept in the inverted position until they began to rouse from the anesthetic.

The procedure employed in harvesting the tissues was as follows: The animals were exsanguinated by cutting the neck vessels with a sharp razor, immediately after which the tissues were fixed *in situ* by gentle perfusion with the fixative. About 20 cc. of the fixative was first introduced via one carotid, and then a similar amount via the other, using a 20 cc. Luer syringe with a 22 gauge needle. Of a number of fixatives initially tried, the one which proved most satisfactory with the staining procedures employed was Bouin's solution. It was found that the perfusion must be carried out gently enough not to separate the olfactory mucosa artificially from the lamina propria or to otherwise disrupt the tissue. Preliminary perfusion with saline solution was not found to improve the fixation or the staining results, and, if anything, impaired the results. Its use was therefore abandoned early in the work.

After the perfusion, the soft tissues from both sides of the nasal vault were removed as one connected mass, usually with the olfactory nerve fila and olfactory bulbs included. To accomplish this satisfactorily required practice. The first step consisted of carefully breaking away small portions of the frontal and nasal bones with a bone forceps of suitable size. After the area had been adequately exposed, it was a relatively simple matter to detach the lamina propria from its bony attachments and to extract the mucosa *in toto* from both sides, along with the olfactory nerve fila and olfactory bulbs. To accomplish removal of the latter two as part of the interconnected mass, it was necessary to remove the bony spicules carefully within the olfactory foramen, the homologue of the cribiform plate in man.

After its removal, the tissue mass was reoriented on a glass slide to bring the parts back as nearly as possible into their normal relationships.

Fixation in Bouin's solution was continued for one or more days, following which the tissue was put in 35 per cent alcohol, and from there carried through graded alcohols. The tissues were finally blocked in paraffin. Sections were cut as thinly as possible (5 μ or less) through the coronal plane of the normally oriented tissue, and mounts were made from different levels through all or most of each mucosa. This orientation made

TABLE I

SPECIMENS OF NASAL MUCOSA

Stage of repair	Mucosal specimens examined (no.)	Stage of repair	Mucosal specimens examined (no.)	Stage of repair	Mucosal specimens examined (no.)
Controls*	48	14 days	3	29 days	3
1 day†	13	15 days	4	30 days	7
2 days	11	16 days	3	31–40 days	10
3 days	11	17 days	4	41–50 days	7
4 days	9	18 days	2	51–60 days	10
5 days	6	19 days	2	61–70 days	5
6 days	3	20 days	4	71–80 days	7
7 days	4	21 days	7	81–90 days	4
8 days	5	22 days	3	3–4 months	25
9 days	6	24 days	2	4–5 months	27
10 days	9	25 days	5	5–6 months	4
11 days	4	26 days	5	6–7 months	11
12 days	1	27 days	1	7–8 months	11
13 days	10	28 days	5	12 months	1

* Control animals were untreated.

† "Days" indicate interval following intranasal irrigation with zinc sulfate solution.

possible observations on all 4 surfaces of the mucosa, at different levels, with portions of the respiratory mucosa often included. Generally included also in the sections were olfactory nerve fila, olfactory bulbs, adjacent meninges, and a small portion of the frontal lobe of the brain.

The staining procedure found most satisfactory and used almost exclusively was that described by Bodian [8,9] for staining nerve fibers. Although various modifications were tried during the early period of these studies, we did not find any of these superior. An essential ingredient in the reagents employed was "Protargol," supplied at that time by the

Winthrop Chemical Company for "tissue staining only," a product which we understand is no longer available. We have since tried protargols provided by the same company for medicinal use without obtaining successful results. With careful observation of the details of the procedure, the Bodian method with the protargol above referred to yielded excellent results on Bouin's fixed material. However, inadvertent overfixation with Bouin's solution at one time caused the loss of a large amount of valuable material.

The total number of specimens of olfactory mucosa from normal and treated monkeys that were successfully processed and studied cannot be stated definitely, but can be said to exceed 300 in number, which, with at least 10 sections per specimen, would mean at least 3,000 sections. Actually, the number of slides examined, usually with 4 or 5 sections per slide, greatly exceeded this figure, since an average of considerably more than 10 slides with "repeats" for better staining results, was the rule. A partial list of the total number of specimens examined and their distribution from the standpoint of the stages of repair represented is given in Table I. As indicated, 48 of the specimens came from untreated, normal monkeys; 68 from animals treated with zinc sulfate solution less than 10 days previously; 84 from animals treated 10 to 30 days previously; 27 treated from 31 to 60 days previously; 16 treated from 61 to 90 days previously, and 79 treated from 3 months to one year previously. The greatest attention was given to mucosa treated 3 months or more previously, after which time animals had shown themselves resusceptible to the neuronotropic virus employed.

Observations

Since the structure of the normal olfactory mucosa is not the primary theme of this report, this will be dealt with only insofar as certain facts relating to it have a bearing on this study. The epithelium is a fairly thick structure composed of olfactory cells (sensory neurons), of columnar shaped sustentacular or supporting cells, and of basal cells, usually rather squat, the whole presenting the appearance of a pseudostratified epithelium (Figs. 1 to 4). This structure, it is important to note, rests directly on the lamina propria, and is not separated from it by a basement membrane. In the lamina propria are found the glands of Bowman, ducts from which open at the surface of the mucosa, providing a serous secretion. Also found are bundles of axons from the olfactory cells which, soon after emergence from the epithelium, become ensheathed by Schwann cells; these constitute unmyelinated nerve (Fig. 1).

Normally, the bodies of the olfactory cells lie mostly in the midzone of the epithelium, but may at times be seen either near its base or near

its surface. The axons of the olfactory cells occur as fine, threadlike processes of uniform diameter which course between the cells of the mucosa in a winding or undulating manner to reach the lamina propria. Here they become grouped into bundles of increasing size which become ensheathed by Schwann cells soon after reaching the lamina propria. The dendritic process is usually thicker and more variable in form than the axon. It may be bayonet-like, its point surmounted by a short, delicate hair (olfactory cilium); it may be in the form of a thick process, solidly stained or lacy, in either case with bulbous or fingerlike terminals surmounted by one or more olfactory hairs, or may show other variations in form (Figs. 2 to 4). It has been postulated that differences in odor perception might be related to differences in function of the individual sensory elements. Should this be true, and differences in function be associated with differences in structure as well, these could be accounted for, to some extent, by the differences in form we have observed. The ends of the dendrites come close to the surface of the mucosa, while the olfactory cilia extend slightly beyond the surface, where, as has been stated, they are bathed by serous secretions from the glands of Bowman.

In the absence of either a dendritic or axonal process, there is relatively little to enable one to differentiate olfactory cells definitely from sustentacular cells by the staining method we employed. While certain differences in the nuclei and cytoplasm, and in staining properties, may be observed, we have felt unwilling to identify cells as olfactory cells definitely unless either an axon or dendrite was seen to be associated with it, or to identify a group of cells as containing olfactory cells unless dendrites or axons were associated with the group. Undoubtedly, many incompletely differentiated olfactory cells were observed during the course of these studies, but we preferred to limit ourselves to criteria of which we could be certain.

This report deals exclusively with the repair of the olfactory epithelium in monkeys, following its destruction by zinc sulfate. Thoroughly applied, a one per cent solution of this agent causes coagulation necrosis involving the full depth of the epithelium down to the lamina propria, and usually most or all of its entire area. Small areas may be missed at times, but these can be easily identified by their normal structure. The respiratory epithelium is little affected.

The extent of the damage inflicted became evident on histologic examination 1, 2, or 3 days after application of the solution, during which time the necrotic epithelium was found in the process of peeling from the lamina propria. It usually came off *en masse* as a coherent membrane, often with many nuclei preserved by the fixative action of the solution (Figs. 5 to 8), the lamina propria being spared. Separation generally

took place first at the arch of the nasal vault, where it was most easily observed in sections cut in the coronal plane, but could be observed to take place also from other locations in suitable sections. It is important to add that its separation was usually so sharply defined and complete as to be comparable to a neat dissection. This has an important bearing in considering the repair that followed. Beginning separation at the top of the nasal vault could be observed in about 24 hours and was usually completed everywhere in 3 or 4 days. However, delayed separation in some places was observed in exceptional cases as long as 8 days after treatment.

As the separation proceeded from the vault downward, a single layer of new flat cells followed closely (Figs. 6 to 8). These cells increased rapidly in number so that by the third or fourth day the thickness of the new epithelium equaled or exceeded that of the normal. The cells making up this epithelium were derived largely if not entirely from the lamina propria, and with the possible exception of those portions bordering on the respiratory mucosa, did not arise from the latter, as reported by Smith [10] in rats. In other words, the new epithelium, with minor exceptions only, was not ciliated. In its early development, it resembled an atypical pseudostratified epithelium, with cells piled up in considerable disarray (Fig. 9). The thick new epithelium was often associated with marked proliferative activity in the lamina propria as well, evidenced by cords of cells extending from the new epithelium into the lamina propria (Fig. 9). At least part of the new cells were derived from duct cells of the glands of Bowman, some possibly from the sheath cells of Schwann, and some possibly from other elements in the lamina propria. The distinctive character of the new epithelium tended to be retained for some time, but there was also a well defined tendency for the cells to orient themselves gradually as in the normal olfactory epithelium. In some areas the new epithelium might not attain more than 1, 2, or 3 cells in depth and was either ciliated or nonciliated. Such areas, however, were usually comparatively small and few in number.

Prior to the tenth day there was relatively little to suggest that olfactory cells would be restored, although abnormal looking cells with processes, along with what appeared to be degenerated nerve fibers, were found at times during this early period of repair. Such findings were usually interpreted as residua of damaged neurons carried upward by proliferating cells. However, soon after the tenth day, normal-appearing cells with dendritic processes could generally be found in the new epithelium. The dendritic process seemed to be the first of the two processes to form and, from the first, was distinctive enough to be easily identified. The incidence of cells with processes increased sufficiently so that by

the 20th day they were numerous in most areas. While such cells might remain relatively few in number in some portions, their number, after several months, approached those of a normal epithelium in most regions. In certain mucosal fragments, removed 6 months to a year after zinc sulfate treatment, the general structure of the epithelium in most portions of the olfactory area was essentially normal. In certain of these late specimens, the epithelium appeared thicker than normal with a seemingly higher incidence of olfactory cells per unit area. Indeed, were it not for the extent of such portions, compared with the usual extent of the initial damage, it would have been easy to underestimate the probable extent of the initial damage or degree of restoration.

As stated earlier, patches of epithelium sometimes escaped the action of the zinc sulfate solution, presumably because the solution was shunted around the areas in question. These areas, however, were generally easily identified as "missed" and were usually not a problem in observations on the early stages of repair. In the later stages, they were less easily differentiated so that the average state of a given mucosal specimen had to serve as a measure of the probable extent of the repair. It should be added here that even in the late stage of repair, not all of a given mucosa was likely to show a restoration approaching the normal. In some of the later specimens there were one or more areas, usually relatively small, in which the cells were only one or two deep, with few or no olfactory cells included, or with the lamina propria covered by ciliated respiratory epithelium. Explanation of these exceptions was not considered within the scope of an investigation limited to the question of whether or not olfactory cells are restored.

Because space would not allow this, the observations made on the progress of the repair cannot be presented on either a case-by-case or a fixed-time-interval basis. It must therefore suffice to summarize the kinds of observations made which were held to indicate that in the repair, sensory elements (olfactory cells) were also restored. The evidence of this was based on a combination of the following kinds of observation: (a) the character and extent of the initial damage induced by zinc sulfate solution—this constituting a base line for observations on subsequent events; (b) the mode of separation of the necrotic epithelium from the lamina propria, seemingly leaving the surface of the latter free of residual olfactory cells (Figs. 5 to 8); (c) the character of the immediate re-epithelization of the denuded lamina propria with cells predominantly derived from it rather than from the adjacent respiratory epithelium (Fig. 9); (d) the appearance of distinctive individual cells or of compact nests of cells, associated or not with nerve fibers, in the new epithelium (Figs. 10 to 22); (e) the increased incidence of cells with dendritic

processes after the third or fourth week of repair; (f) the exceptional location of individual olfactory cells in newly formed epithelium, such as cells found close to the surface of a new epithelium (Figs. 10, 11, 17); (g) abnormalities in the orientation, grouping and general distribution of olfactory cells in late epithelium retaining some of the earmarks of having regenerated; (h) the extent to which an essentially normal epithelial structure was found after several months (Figs. 23 and 24); and (i) the resusceptibility of monkeys to intranasal inoculation with a neuronotropic strain of poliomyelitis virus 3 or 4 months after intranasal treatment with zinc sulfate solution. As reported by Schultz and Gebhardt,[2] this resusceptibility was associated with anatomic evidence of axonal regeneration and with the usual lymphocytic infiltration accompanying neuronal transmission of the virus.

Certain odd occurrences of olfactory cells were observed in specimens obtained from late stages of repair. Among these was the appearance of gland-like spaces lined in part by olfactory cells (Figs. 25 and 26), spaces seemingly formed by invaginations of portions of the olfactory mucosa (Fig. 27). In these the overlying surface epithelium consisted of either essentially normal olfactory epithelium, or of either ciliated or nonciliated cells, without the presence of olfactory cells. A similar occurrence was the presence of islets of compact olfactory cells below a surface epithelium, the latter being either of normal olfactory structure (Fig. 28) or not. How these might have arisen was not clear. They could have arisen from either a burial of surface cells during the rapidly proliferating stage of the repair, or developed from potential olfactory cells in the depth of the tissue.

Still another unexplained observation made on membranes in the late stages of repair was the occasional occurrence of short stretches of nonciliated epithelium made up of somewhat cuboidal cells one or two cells in depth, at times with an occasional squat olfactory cell included. It would be of interest to know why such highly abortive restorations occurred in the midst of more or less fully repaired olfactory epithelium. Without knowledge of the usual extent of the initial damage, one might conclude that these represented the full extent of the original damage, a conclusion that would be at variance with the many observations made on membranes early in repair.

Discussion

The observations reported here seem to justify the conclusion that in the repair of the olfactory mucosa following zinc sulfate-induced necrosis, sensory neurons (olfactory cells) are largely restored. The question posed in these studies was not whether complete restoration occurred,

but whether or not restoration occurred at all. There was a basic reason for asking this question. In the olfactory cell we have a primitive type of neuron represented whose regenerative capacity might differ from that of other neurons in vertebrates. Prior to our first reports,[1,2] no evidence had been recorded indicating that the loss of olfactory cells could be followed by restoration.

The fact that areas were found in membranes late in repair, in which the restoration of olfactory cells was either slight or absent, constitutes, we believe, a separate problem, one relating to the factors that restrict the extent of restoration. Another problem considered to lie outside the scope of the present paper was the precise origin of the olfactory cells. While this question was of interest, it was held for possible future studies.

In 1938, Smith,[10] on the basis of observations made on rats, following intranasal application of zinc sulfate solution, reported that the destroyed olfactory epithelium was replaced by ciliated respiratory type of epithelium only, and that specimens obtained up to two months after treatment failed to show a replacement of olfactory cells. In 1951, however, Smith[11] reported observations that led him to the conclusion that a regeneration of "sensory olfactory epithelium" did occur. The latter observations were made on adult frogs in which the olfactory epithelium had been either destroyed by zinc sulfate solution or removed by operation. However, it should be noted that in neither of the two above investigations did he use other than the hematoxylin and eosin stains in his sections. Success in demonstrating nerve fibers of olfactory cells by simple staining methods is at variance with our own early experience, and if this were possible, would have saved us an enormous amount of technical work. On the contrary, it has been our experience that the fibers of olfactory cells are more fastidious, if anything, in their reception of staining methods than are the nerve fibers in the olfactory bulbs, with which staining results could be compared in the same sections.

Undoubtedly, many olfactory or potential olfactory cells, still without fibers, were not identified or classified as such in the specimens examined, since we wished to keep on secure ground and consider only cells with definite nerve processes. Where dendrites were numerous in a given area, we could assume that nerve cell bodies also were numerous. The reverse, however, was not necessarily true, since some olfactory cell bodies might not yet have formed fibers.

Just how early in a regenerating mucosa recognizable olfactory cells appear, we cannot state definitely, but these were observed soon after the tenth day. It is also not possible to state how long the restoration may continue, but the impression gained is that it may continue over a period of several months. Although mitotic figures were observed in the

regenerating epithelium, these were not numerous and were not identified with distinctive cell nests. On the other hand, the appearence of distinctive paired cells, and of closely packed cells in cell nests suggested that amitotic division may occur commonly.

Summary

Observations are reported on the repair of the olfactory epithelium in rhesus monkeys following coagulation necrosis induced by zinc sulfate solution. These observations show that in the repair of the epithelium, olfactory cells (sensory neurons) are largely restored. The restoration of these cells is seemingly a gradual process, extending over a period of several months, but may end in a restitution of most of the epithelium to its normal state within 6 months to a year. There are unexplained exceptions in some areas.

References

1. Schultz, E. W. Regeneration of olfactory cells. *Proc. Soc. Exper. Biol. & Med.*, 1941, **46**, 41–43.
2. Schultz, E. W., and Gebhardt, S. P. Studies on chemical prophylaxis of experimental poliomyelitis. *J. Infect. Dis.*, 1942, **70**, 7–50.
3. Geist, F. D. Nasal Cavity, Larynx, Mouth and Pharynx. In: The Anatomy of the Rhesus Monkey (*Macaca mulatta*). By Bast, T. H., Christensen, K., and others. Hartman, C. G., and Straus, W. L., Jr. (eds.). Williams & Wilkins Co., Baltimore, 1933, Chapter 9, pp. 189–209.
4. Schaeffer, J. P. The Nose, Paranasal Sinuses, Nasolacrimal Passageways, and Olfactory Organ in Man; a Genetic, Developmental and Anatomico-physiological Consideration. The Blakiston Co., Philadelphia, 1920, 370 pp.
5. Parker, G. H. Smell, Taste and Allied Sense in the Vertebrates. J. B. Lippincott, Philadelphia, 1922, 192 pp.
6. Hopkins, A. E. Olfactory receptors in vertebrates. *J. Comp. Neurol.*, 1926, **41**, 253–289.
7. Odors and the Sense of Smell, a Bibliography 320 BC–1947. Airkem, Inc., New York, 1952, 342 pp.
8. Bodian, D. A new method for staining nerve fibers and nerve endings in mounted paraffin sections. *Anat. Rec.*, 1936, **65**, 89–97.
9. Bodian, D. The staining of paraffin sections of nervous tissues with activated protargol. The role of fixatives. *Anat. Rec.*, 1937, **69**, 153–162.
10. Smith, C. G. Changes in the olfactory mucosa and olfactory nerves following intranasal treatment with one per cent zinc sulfate. *Canad. M.A.J.*, 1938, **39**, 138–140.
11. Smith, C. G. Regeneration of sensory olfactory epithelium and nerves in adult frogs. *Anat. Rec.*, 1951, **109**, 661–671.

The author is indebted to Mr. Robert Peck for technical assistance in this work.

[*Illustrations follow*]

Legends for Figures

Except where otherwise stated all sections were stained by the Bodian method for nerve fibers. The words "after treatment" used in the descriptions below mean "after intranasal irrigation with zinc sulfate solution."

FIG. 1. General structure of the normal olfactory mucosa. Shown are the relative thickness of the olfactory epithelium and the content of the lamina propria, including glands of Bowman and an olfactory nerve bundle. × 135.

FIG. 2. Dendrites in a normal olfactory epithelium, showing a wavy course through the epithelium and bulbous terminations (olfactory vesicles). The latter end with fine olfactory hairs not clearly visible in the illustration. × 630.

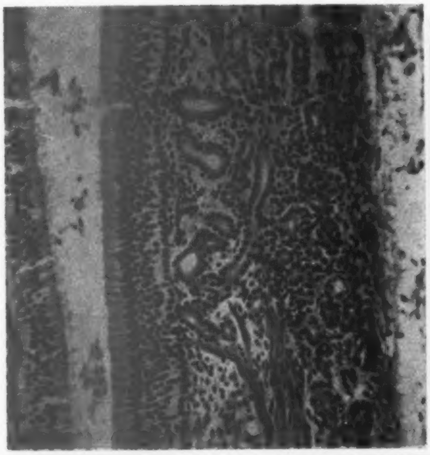
FIG. 3. Dendrites in a normal olfactory epithelium, showing a wavy course through the epithelium, with fingerlike terminations of some and bulbous terminations of others. The former as well as the latter end with fine olfactory hairs. × 1250.

FIG. 4. Some of the variations in the form of dendrites in a normal olfactory epithelium. × 1250. (Reproduced from the *Journal of Infectious Diseases,*[8] with permission of the University of Chicago Press.)

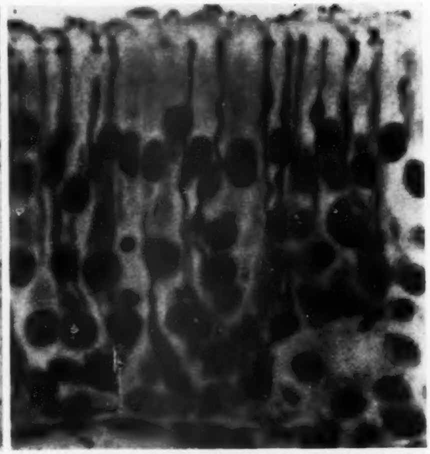
FIG. 5. *En masse* separation of necrotic olfactory epithelium from the lamina propria; near the nasal vault; 28 hours after treatment. × 135.

FIG. 6. *En masse* separation of necrotic olfactory epithelium from the lamina propria; near the nasal vault; 2 days after treatment. A layer of new epithelial cells has begun to cover the denuded lamina propria. Hematoxylin and eosin stain. × 135.

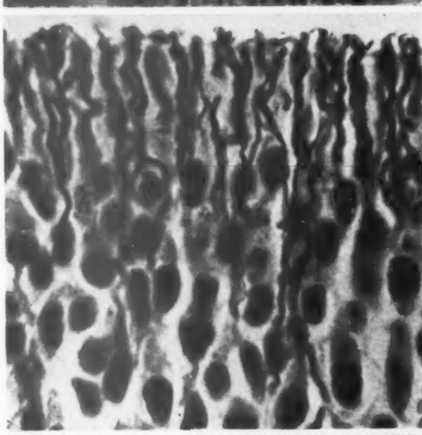
1



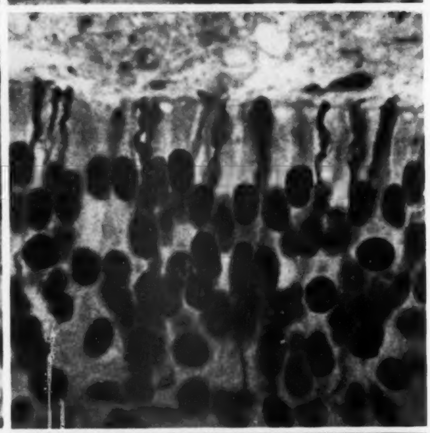
2



3



4



5

6

FIG. 7. Separated necrotic olfactory epithelium lying free in the nasal meatus; near the nasal vault; 44 hours after treatment. A layer of new epithelial cells has formed to cover the denuded lamina propria. Hematoxylin and eosin stain. × 135. (Reproduced from the *Jounral of Infectious Diseases,*[2] with permission of the University of Chicago Press.)

FIG. 8. A layer of new epithelial cells following closely on a separating necrotic epithelium; 44 hours after treatment. Hematoxylin and eosin stain. × 675. (Reproduced from the *Journal of Infectious Diseases,*[2] with permission of the University of Chicago Press.)

FIG. 9. New epithelium 5 days after treatment. A thick epithelium with fingerlike extensions into the lamina propria is manifest. There is no evidence of cells with fibers. × 225.

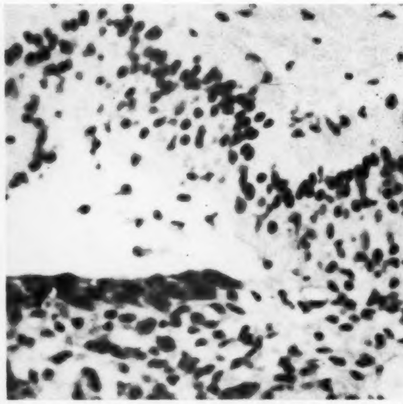
FIG. 10. A lone olfactory cell with a dendrite near the surface of a portion of new epithelium; 17 days after treatment. × 675.

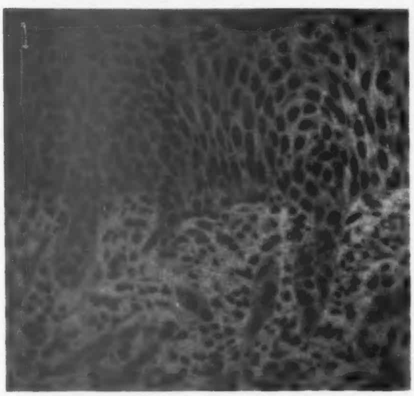
FIG. 11. An oval olfactory cell with a short dendrite near the surface of a new epithelium; 24 days after treatment. This cell is associated with dendritic processes from other olfactory cells. × 675.

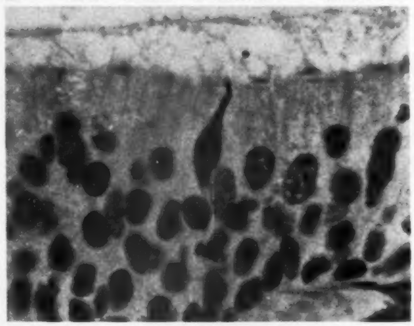
FIG. 12. Scattered, darkly stained cells with fibers in a new epithelium; 22 days after treatment. × 675.

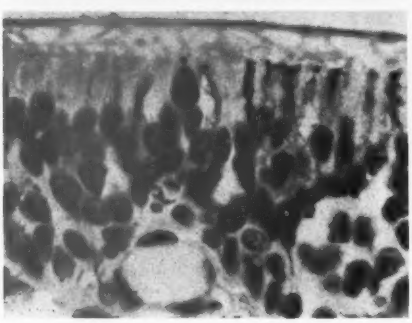
FIG. 13. A mixture of cells and fibers, and a mitotic figure, in a portion of new epithelium; 13 days after treatment. × 1215.

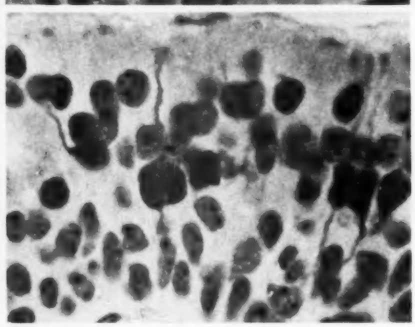

7


8


9


10


11


12

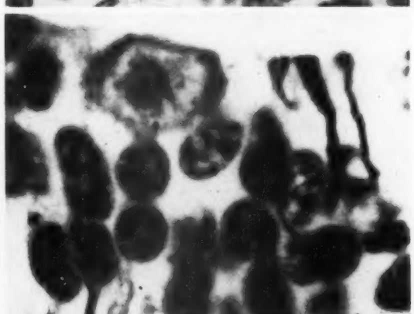

13

FIG. 14. Darkly stained cells and cell masses, associated with dendrites of different form, in a portion of new epithelium; 28 days after treatment. × 1215.

FIG. 15. A nest of cells in new epithelium. A dendrite extends to the surface; 17 days after treatment. × 810.

FIG. 16. A nest of cells in new mucosa. Several dendrites and axons have emerged; 13 days after treatment. × 1215.

FIG. 17. A collection of well separated new olfactory cells with short, plump dendrites, mostly near the surface of the epithelium; 13 days after treatment. × 1215.

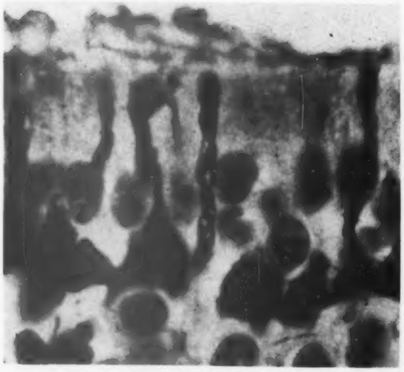
FIG. 18. Cell nests in a portion of new epithelium; 28 days after treatment. These nests are associated with dendritic processes. Some of the new epithelium is ciliated. × 200.

FIGS. 19 and 20. Portions of new epithelium, showing cell nests with dendritic processes; 28 days after treatment. Fig. 19: × 620. Fig. 20: × 1090.

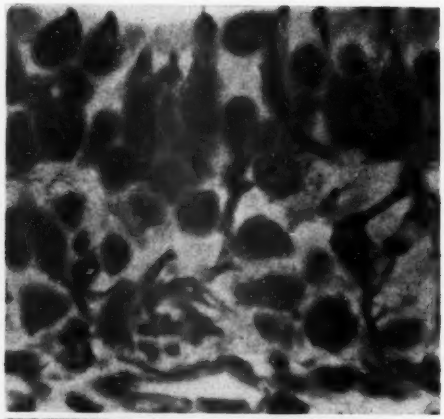
14

15

16

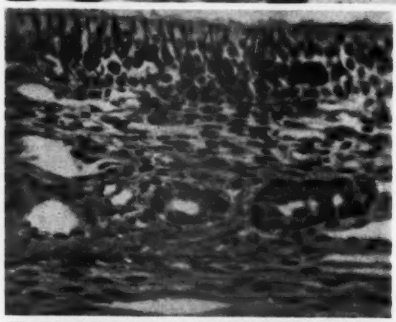


14



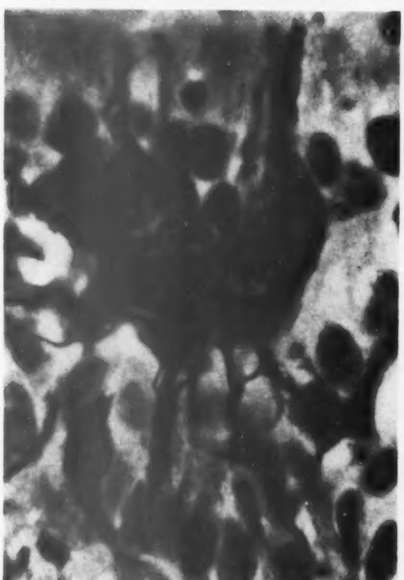
17

15

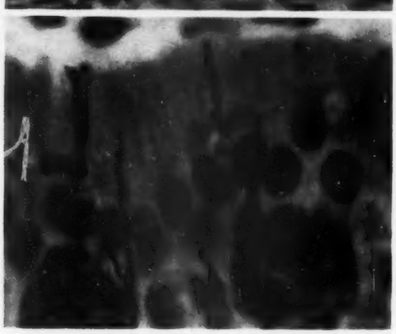


18

19



16



20

FIGS. 21 and 22. Portions of new olfactory epithelium, showing separated cells with dendritic processes; 20 and 21 days, respectively, after treatment. × 810.

FIG. 23. A portion of olfactory epithelium, representative of much of the olfactory area; 4¾ months after treatment. The epithelium is thick, normal in cell arrangement, with about the normal number of dendrites per unit area. × 270.

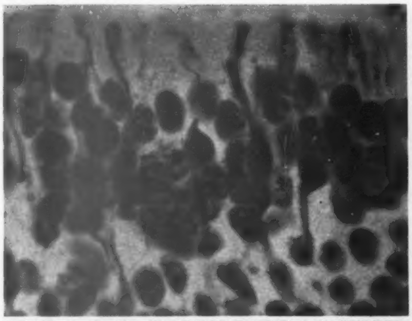
FIG. 24. A portion of olfactory epithelium, representative of much of the olfactory area, from an animal 7½ months after treatment. A thick, essentially normal olfactory epithelium is evident. Basal cells are columnar, instead of squat, as commonly observed in the normal state. × 270.

FIG. 25. A glandlike space, lined in part by olfactory cells, below a surface epithelium; 5 weeks after treatment. Some of the cells of the surface epithelium are ciliated, but none show dendrites. × 210.

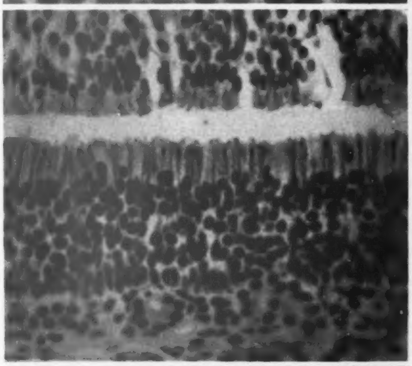
FIG. 26. Glandlike spaces, lined in part by olfactory cells, lie below the surface epithelium; from an animal 3½ months after treatment. No olfactory cells are present in the surface epithelium. × 210.

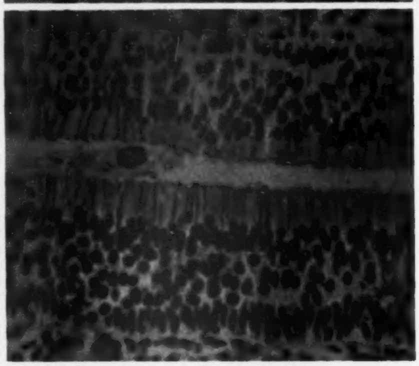
FIG. 27. An invagination of new epithelium, showing how glandlike spaces lined in part by olfactory cells might be formed; from an animal 3½ months after treatment. Surface epithelium is not ciliated and shows no cells with dendrites. × 210.

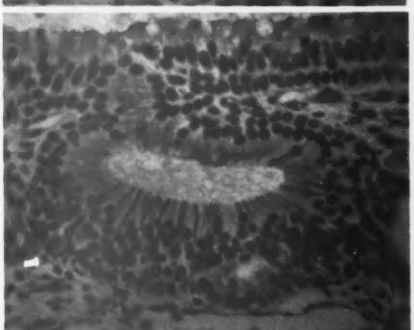
FIG. 28. A rounded mass of olfactory cells below a surface olfactory epithelium that is essentially normal in structure; from an animal 4¾ months after treatment. × 270.

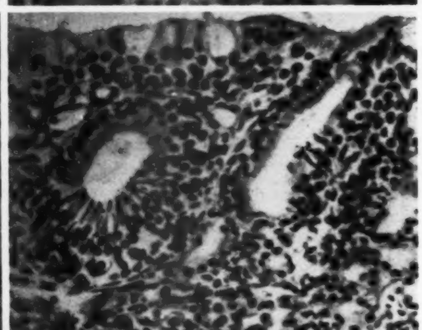
21


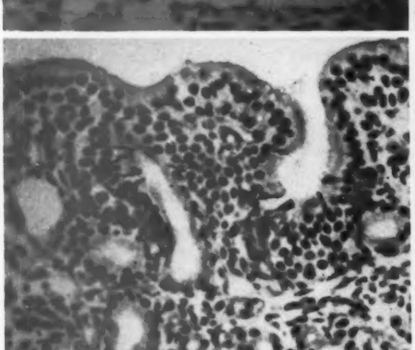

22

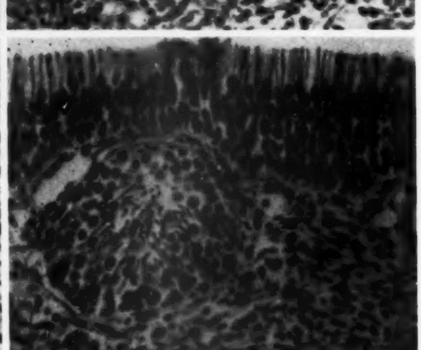
23



24

25



26

27



28

PRECISION IN THE CLASSIFICATION OF CIRRHOSIS OF THE LIVER

PAUL E. STEINER, PH.D., M.D.

From the Institute for Cancer Research, Philadelphia, and the Department of Pathology, School of Medicine, the University of Pennsylvania, Philadelphia, Pa.

There is now wide agreement on the morphologic classification of the main types of cirrhosis, even though some differences of opinion remain on which synonyms are best and on pathogenesis. The classification of the special types such as biliary, central, pigmentary, parasitic, and others usually poses no difficult problem. Interesting as these types are, they are not a major part of the cirrhosis problem in most parts of the world. In the large group, two types have been recognized by many authorities and by international conferences.[1,2] These are postnecrotic and portal cirrhosis. If these concepts are conceded, the problem remains of precise distinction between them because they do merge. Using a different terminology, Baggenstoss and Stauffer recently compared 43 cases each of posthepatitic and alcoholic cirrhosis and found considerable overlap in the appearances.[3]

A classification of cirrhosis which could be used in surveys was recently needed.[4] It was to be used to permit the comparison of series of cases in one geographic area, ethnic group, and institution, with others. The method had to meet the following requirements: to be as objective as possible; to have relatively simple and easily definable groups; to accommodate all cases; to express quantitative as well as qualitative differences; to make as much use of existing terms as possible; to give reproducible results in different hands.

Classifications can be etiologic, pathogenetic, prognostic, functional, or morphologic. Because the etiology and the pathogenesis are not known in most cases of cirrhosis, they could not properly serve as the basis for comparison in such an investigation. Neither could prognosis or functional derangements be used because these data would usually not be available. Objectivity being thought essential, the use of etiologic terms such as "nutritional" and "alcoholic" was excluded even though the ultimate objective of the studies was to shed light on causation. A classification based on pathogenesis was also not acceptable because the

Aided by a research grant (C 3251) from the National Cancer Institute through the University of Chicago.

Received for publication, October 2, 1959.

genesis of the lesions would be unproved, except in those few cases in which serial liver biopsy specimens had been procured or in those in which the mechanism of the origin of the lesion happened still to be apparent in the end product. If what is equally visible to the eyes of all observers was to be used as evidence, the classification would have to be morphologic and descriptive. Then, in the future, as structure became accurately correlated with etiology and pathogenesis, the data, being objective and precise, could be reassessed in those terms.

The classification of the Havana conference was adopted,[1] and ways were sought which would sharply and precisely distinguish postnecrotic and portal cirrhosis, while meeting the requirements mentioned above. Accordingly 412 cases of cirrhosis in the files of the Department of Pathology, the University of Chicago, were restudied. (In 191 of these cases, the cirrhosis was mild or early and not the cause of death, but it provided information on genesis.) Data were recorded in each case on about 30 microscopic features. A sample of this data sheet is published elsewhere.[4]

From this study two main findings emerged: (1) It was possible to draw a sharp, objective, and defensible line between the postnecrotic and portal types of cirrhosis. (2) It became desirable and easy to subdivide portal cirrhosis into 4 subgroups in the interests of a more precise characterization. Also, a category—florid cirrhosis—was added for reasons that will become apparent; it was later found to have an interesting ethnic and geographic distribution.[4]

This method of classification was then used in Africa in 1957 in 9 collections of cirrhosis, in which 943 cases represented 8 different geographic and 9 ethnic groups. The method was again found to be simple, yet sensitive enough to reveal group differences.[4–6]

It is the purpose of this paper to explain this classification and its use. It is emphasized that the classification is purely microscopic and morphologic although it correlates well with the gross appearances. Also, although some of the terms could have an etiologic or a pathogenetic meaning, usage, or connotation, this fact is coincidental, and they are used in this classification strictly in a morphologic sense.

Description of the Method

The classification is given in Table I. In its use, the microscopically distinctive types are readily recognized and separated on the basis of accepted old and established criteria. These rather clear-cut types include pigmentary, biliary, central, parasitic, and the miscellaneous forms of cirrhosis. Some of these can be subclassified according to the well known criteria available in the literature.[7–9] This leaves 3 types in any

collection, of which 2—postnecrotic and florid—are precisely recognized on the basis of the criteria to be given. The bulk of the livers in some groups of cases still remain. They are found essentially to comprise one variety, having variations. This type is portal cirrhosis, which is subclassified into 4 rather distinct groups. Although it remains in one's thinking until the last, portal cirrhosis is not a dumping ground but a distinct category having definite characteristics.

TABLE I

CLASSIFICATION OF CIRRHOSIS

Postnecrotic cirrhosis
Portal cirrhosis:
Type A (simple)
Type B (simple, with areas of questionable collapse)
Type C (simple, plus fat)
Type D (simple, plus fat and intranodular fibrosis)
Florid cirrhosis
Pigmentary cirrhosis:
(a) Obstructive
(b) Infectious (cholangitic)
(c) Other
Central (cardiac and others)
Parasitic cirrhosis
Mixed cirrhosis
Miscellaneous types
Unknown types

General Concepts in Cirrhosis

Throughout this study the term cirrhosis has been used for the generally nodular livers that microscopically exhibit fibrous bands, membranes, septums or scars which subdivide the organ into nodules and, together with nodular hyperplasia or regeneration, distort the original hepatic architecture. The two components, new fibrous tissue and some degree of nodular parenchymal hyperplasia, jointly cause the structural abnormality and are both essential. This definition is in essential agreement with that of the Havana[1] and Kampala conferences.[2]

This definition simplifies the cirrhosis picture by eliminating two confusing conditions, namely "hepatofibrosis" and "nodular regenerative hyperplasia."[10] Hepatofibrosis includes all of the types of fibrous tissue increase not accompanied by nodular regeneration or hyperplasia. It may be localized to one part of the organ, or it may be generalized throughout the liver but focal within the lobules. Three examples are the early stages of the central fibrosis of chronic passive hyperemia, the periportal fibrosis of chronic biliary tract obstruction, and the random focal fibrosis of schistosomal granulomas. In none of these examples is the fibrosis septal, linear, or connected at this stage; nor is there ap-

preciable nodular regeneration, so they fail to meet the requirements for cirrhosis. The interesting and uncommon condition of nodular regenerative hyperplasia, on the other hand, does not meet the definition of cirrhosis because of the absence of fibrosis. It has undoubtedly been classified as cirrhosis by some, and the suggestion has been made that for reasons of statistical accuracy it be separated out.[10]

The new fibrous tissue of cirrhosis may be diffuse or focal, but at least some of it is linear. These new fibrous structures have variously been designated bands, septums, membranes or scars.[11] Their appearance may vary according to the plane at which the section happens to strike them. In this paper these terms are used interchangeably.

The parenchymal nodularity is caused by the delimitation produced by the fibrous tissue and by hepatocytic hyperplasia or regeneration. If hyperplasia is great, the ability to distinguish it from regeneration becomes difficult. Although they represent different pathologic processes, in this paper no attempt has been made to separate them. Both result in atypical liver tissue. The important question whether regeneration can ever be so perfect as to produce histologically normal-appearing liver has not been answered. In this paper it is assumed that regenerated liver is always atypical histologically, and usually cytologically as well.

To avoid confusion, the term "lobule" is used for the normal anatomic lobule, and the term "nodule" is used for the nodular units in cirrhosis, regardless of whether they consist of only a part of one or of many anatomic lobules or of new abnormal parenchyma, the result of regeneration or hyperplasia.

Classification

In this section postnecrotic, portal, and florid cirrhosis are defined, described, and illustrated. The other types are recognized according to the standard criteria given in the literature so that it is not necessary to describe them here.

Postnecrotic Cirrhosis

(Post-collapse cirrhosis; posthepatitic cirrhosis; toxic cirrhosis; healed acute yellow atrophy; healed hepatic necrosis; etc.)

Postnecrotic cirrhosis is that type which shows abnormal accumulation of stromal components such as portal tracts, hepatic veins and sinusoidal fibrous tissue, in its fibrous scars, bands or septums. The condensation and juxtaposition of these stromal elements represent the collapse of one or more anatomic lobules after the loss of their parenchyma by necrosis or by atrophy with resorption. The original necrosis is now not evident, but the collapse is. These features are illustrated in

Figures 1 to 9. The term "post-collapse" would, therefore, be more fitting than postnecrotic cirrhosis, but the latter is widely established, so it is retained in the interest of harmony. The diagnosis of postnecrotic cirrhosis is made when the microscopic examination discloses the presence of 3 or more portal triads abnormally collected in the fibrous scar tissue at one or more places in the sections. By definition, regeneration or hyperplasia of hepatocytes is also present. It is emphasized that the designation of postnecrotic cirrhosis is a morphologic one, in each case based on what is actually seen, even though the term also expresses the pathogenesis of the lesion.

In postnecrotic cirrhosis the fibrous areas are usually large in some places but slender and long in others (Figs. 1 to 3, and 7). They are large enough to contain from 3 to a dozen or more portal areas in one plane through some scars, but thinner bands of connective tissue may connect such areas or pass out into the nodules (Figs. 1, 2 and 4 to 6). The scars, septums, bands, or membranes are formed in part by the condensation of stroma (Figs. 8 and 9), but they may be enlarged and made more dense by the addition of new connective tissue. Therefore, depending on their age and origins, the fibrous areas may be loose or dense, small or large, short or long, and poor or rich in nuclei, including those of the cells of chronic inflammation.

In the scars, the portal tracts are easily recognized as such when the cirrhosis is young and the collapse recent (Figs. 4 to 6). The 3 triadal structures are still enclosed by dense connective tissue which is in part concentrically arranged as in the normal tract, labeling the structure for what it is (Fig. 5). They are separated from the other triads in the scar by fibrillar tissue which, for a time, is loose. With silver or sensitive connective tissue strains, this is seen to represent chiefly the sinusoidal stromal elements which have become condensed because of the disappearance of the hepatocytes and collapse of the cords (Fig. 8). The hepatic veins also become incorporated into the scars by the collapse and condensation, but they are harder to recognize because of the absence of distinctive features.

In differential diagnosis these portal tracts, abnormally brought together, need be distinguished only from portal tracts at points of normal anatomic bifurcation. The distinction is based chiefly on the relations and appearance of the tissue enclosing the tracts—whether it is normal liver on the one hand, or new scar tissue or condensed stroma on the other. Moreover, more than two normal bifurcating triads connected in the section are rarely seen.

With the passage of time the triads in the scars become progressively harder to recognize as such. Progressive distortion by the accretion of

collagen, and by retrogressive changes, such as atrophy of the epithelium of the bile ducts, endosclerosis of the blood vessels, and fusion of the collagenous elements, eventually results in scars in which the formerly organized elements disappear, and the distinctive features of triads and collapse are lost (Fig. 7). Fortunately, many livers contain scars in various stages of evolution.

In postnecrotic cirrhosis the liver nodules are usually large, but they may be small or mixed. They may contain original or regenerated liver tissue or both. Large nodules may contain a number of lobules, and little ones, as few as one or even less. Thus the nodules, and the cirrhosis, may be either monolobular, multilobular, or mixed—using those terms in the sense of the original anatomic hepatic lobules. Fat is not conspicuous; it is usually absent to slight, although occasional nodules in some cases may contain appreciable amounts. The fibrous areas sometimes contain large, thin-walled blood vessels regarded as "sinusoidal varices" in addition to the portal and hepatic veins and the hepatic arteries. These varices are enlarged sinusoids which have become incorporated into the scar. Biliary tract hyperplasia is inconspicuous except when biliary obstruction has been added. Thus the liver of postnecrotic cirrhosis is usually an irregularly nodular organ which tends to be reduced in size. The picture, however, is variable, and the only constant feature is the accumulation and approximation of portal tracts in the scars.

Examination of the changes in greater detail reveals that the juncture of the connective tissue septums and the liver cells may, variously, depending on the stage and activity of the process, be sharp and abrupt, or indistinct because of an inflammatory reaction and liver cell degeneration, or irregular because of extensions of fibrous tissue into the nodule (Figs. 1 to 3). Proliferation of structures resembling small bile ducts, cholangioles, or cholangiole-like cells may be present within the connective tissue septums, at the junction of liver cells and septums, or occasionally, within the liver nodules themselves (Figs. 4 to 6). An inflammatory reaction is always present, but it varies greatly in degree, in type, and in location. It may consist of only a few small round cells in the septums or of greater numbers of eosinophils, histiocytes, lymphocytes, plasma cells, and sometimes neutrophils located either in the parenchyma or stroma. Fibroblasts are present though not numerous. The amount of collagen appears disproportionate at all stages to the number of fibroblasts. Evidences of bile accumulation may be present in the form of casts in ducts or canaliculi, or as accumulations in liver or Kupffer cells. This obstruction is often accompanied by a proliferation of the small biliary ducts already mentioned and by bile necroses. Small

islands or groups of liver cells, compact or separated by an inflammatory or fibrous reaction, and showing degenerative changes, are almost invariably found if sought within the large fibrous areas (Figs. 4 and 8). Atypical liver cells are almost always present, occasionally in great numbers. Anisocytosis and poikilocytosis of liver cells, similar variations in the nuclei, their number and their chromatin pattern, and vacuolar or hyaline alterations may all be found. Parenchymal necrosis, as a late or subterminal change, may take the form of bile necrosis or of infarction of entire nodules or parts thereof.

Postnecrotic cirrhosis shows a wide range in gross and microscopic appearances, depending on the age and severity of the process, but the essential change is always collapse of anatomic lobules, usually simultaneously but sometimes over a long period. At one extreme, and recognized most easily, is the liver which shows large scars (up to several centimeters) containing no parenchyma or at most only small groups of cells (Figs. 1, 4 and 5). Here and there are scattered larger nodules of liver tissue, much of which appears abnormal in its cytology and histologic structure and has the appearance of regenerated nodules. The larger nodules may contain some relatively normal-appearing lobules resembling original liver. These livers are small and irregularly nodular. At the other extreme is the larger liver which has a higher component of parenchyma and narrow fibrous septums in which there is evidence of previous lobular collapse only in scattered places (Figs. 2 and 3).

All parts of the liver are involved, but the distribution may be uneven, and some lobes may be disproportionately reduced. Because there is actual loss of liver substance, the organ usually becomes small, and it may be as little as 550 gm. The smallest of all livers fall into this category.

After cirrhosis has been classified as postnecrotic, it may be further characterized in several ways for descriptive and comparative purposes. It may be subclassified as multilobular, monolobular, or mixed. With respect to the estimated degree of activity or speed of the process as judged by the amount of fresh necrosis and by the amount and type of the inflammatory reaction, it may be subclassified as active or quiescent, or this feature may be graded from 1 to 4 plus (+ to ++++). The severity of the process may be also be designated as from + to ++++ on the basis of the amount of fibrosis and regeneration. This is particularly useful in dealing with cases which were not fatal.

The pathogenesis of postnecrotic cirrhosis as being a collapse phenomenon, at least in part, can best be demonstrated in a series of cases, and sometimes in one liver, by the use of stains for reticulin (Figs. 8 and 9) supplemented by those for collagen and for elastic fibers. Once the picture is understood, its subsequent recognition becomes a low-power diagnosis in most instances.

Because the essential change in postnecrotic cirrhosis is visible evidence of loss of parenchyma with collapse and condensation of the stroma, "post-collapse cirrhosis" would be a better term. The other synonyms indicate the uncertainties regarding both the etiology and the pathogenesis of this variety of cirrhosis. Regardless of the cause and nature of the original liver cell necrosis, there often appears to be a continuing process of necrosis, atrophy and inflammation on the basis of circulatory inadequacy within the lobules, or of a continuing damage from other unknown causes.

The synonyms reflect the diversity in both the pathogenesis and the etiology of this type. In most of the cases, however, the cause is as yet unknown.[12] The term postnecrotic as here used is a morphologic term which happens, however, at the same time to characterize the genesis. It provides a reasonably homogeneous group which can later, possibly, be subdivided on etiologic grounds. The cases all give evidence of having had collapse of lobules. They all have, also, chronic hepatitis, but whether this represents the reaction to the presence of an injurious agent or whether it is the reaction to liver injury by other causes is not now known. The presence of many plasma cells, not often emphasized, speaks for an immune process. Until feasible tests for infection, present or past, by the hepatitis virus are available, this group will remain difficult to subdivide on an etiologic basis.

Portal Cirrhosis

(Septal cirrhosis; Laennec's cirrhosis; atrophic cirrhosis; hypertrophic cirrhosis; multilobular cirrhosis; cryptogenic cirrhosis; alcoholic cirrhosis; nutritional cirrhosis; etc.)

Portal cirrhosis is that primary form which is characterized by nodules of liver tissue, at least some of which is regenerated or hyperplastic, separated by fibrous scars or septums which give no proof of having been formed by the collapse and condensation of hepatic stroma—they contain no abnormal collections of portal tracts or hepatic arteries. Both changes jointly result in the distortion of the original lobular architecture (Figs. 10 and 11). The new nodules may be either small or large, depending on whether they represent one, several or many original anatomic liver lobules, and on the amount of regenerated liver tissue that they contain. In this sense, portal cirrhosis also may be monolobular, multilobular, mixed, or even intralobular. Grossly, the nodularity is generalized, fairly uniform, of various degrees of severity, depending on the stage at which it is seen, and of various sizes from small to large. This category includes most of the uniformly hobnail livers in which the intervening fibrous scars are thin.

In short, portal cirrhosis is microscopically distinguished from post-necrotic cirrhosis by having no areas of post-collapse scarring that contain 3 or more portal tracts in juxtaposition.

The portal cirrhosis group can be further subdivided on microscopic bases into 4 subtypes for a more accurate characterization for statistical purposes and, perhaps, etiologic considerations.

Portal cirrhosis, type A, is the simple, usual, or typical variety. It has distinct fibrous bands which subdivide the liver parenchyma into nodules of various sizes. The nodules show various amounts of hyperplasia (Figs. 10 and 11). The resulting picture is one of nodular distortion of the liver of either multilobular, monolobular, or mixed type. All of the histologic features of a postnecrotic cirrhosis may be seen in this type except the scars with excess portal tracts.

Portal cirrhosis, type B, is the same as type A except that its fibrous scars, here or there, have evidence suggestive of excessive numbers of triadal structures without, however, conclusively demonstrating this excess (Fig. 12). Thus there is suggestive evidence of previous lobular collapse but not enough to warrant designating the case one of post-necrotic cirrhosis; the great majority of the fibrous areas show no evidence of collapse.

Portal cirrhosis, type C, is the same as type A or B but it has, in addition, appreciable amounts of fat of the large droplet type in at least some lobules (Fig. 13).

Portal cirrhosis, type D, is the same as type C except that it has, in addition, an appreciable amount of fibrous tissue within the nodules: From the principal fibrous bands or septums, fibrous tissue of a more delicate type extends into the nodules between the liver cells (Fig. 14). This type is usually basically monolobular but with intralobular additions.

Except for the characteristic and special features mentioned, most of the details given for postnecrotic cirrhosis apply also to the 4 portal types.

The term portal cirrhosis has been used for this class for want of a better name, because it is widely used, and because it is undesirable to coin yet another term. There appear to be fewer objections to its use than to any other. It describes a nodular, distinctly cirrhotic organ. The microscopic criteria are reasonably objective and they are definite. The term is broad enough to encompass the 4 subtypes, which are essential if series of cases from one place are to be compared with others.

There is reason to believe that the process does not always begin in the portal tracts, and it is certain that some portal areas are spared in the multilobular type, the fibrous tracts sometimes passing near normal-

appearing triads. Thus it is less truly portal in origin than is biliary cirrhosis, in which every portal tract is clearly and early involved.

"Septal" cirrhosis would do equally well, but it is not widely used and it fails to accommodate the intralobular fibrous component of the D type, which is not "septal." The same criticisms may be made for the term "membranous" cirrhosis. The term "Laennec" cirrhosis is objectionable for this group because it is used in the French, Italian, and Spanish literature in a more restricted sense than in the United States. As used in those countries, it would apply only to some of the cases in types C and D and to none of those in types A and B. The terms "atrophic" and "hypertrophic" cirrhosis are discarded because they refer to gross size and give little indication of the microscopic appearances or processes. The term "cryptogenic" cirrhosis admirably expresses the state of knowledge about cause, but it is not descriptive of the lesion. The etiologic terms "alcoholic" and "nutritional" cirrhosis also carry objections. An alcoholic history may be found in cirrhosis of a number of morphologic types, each of which is seen also in persons who consumed no alcohol. This fact is impressive in some Mohammedan countries where alcohol is not used but cirrhosis of the portal type is rampant.[6] Similar objections may be made to the term "nutritional" cirrhosis. It implies an etiologic relationship which, in man, is not established,[13,14] and it includes a variety of morphologic appearances, each of which occurs also in persons who had no known malnutrition. The terms "monolobular" and "multilobular" are useful in description and characterization, but their use for the main types would have little value. All things considered, it seems best to use the term portal at this time.

Florid Cirrhosis

(Subacute cirrhosis; fatty cirrhosis; etc.)

Florid cirrhosis is characterized by perilobular septums which are usually slight to moderate in width and density and from which spring an extensive but delicate intralobular fibrosis, by a nodular hyperplasia, by a large droplet fatty change which is usually generalized, and by small foci of neutrophils and other leukocytes within the lobules, in addition to the usual inflammatory cells in the septums (Fig. 15). It is a true cirrhosis, as judged by new fibrous tissue, nodular regeneration and distortion of architecture. The fibrous tissue extends out from every portal area and outlines most of the anatomic lobules. In addition, a more delicate fibrous tissue extends from these major septums or membranes into the lobules. The fibrosis is therefore anatomically intralobular as well as monolobular. The amount of liver cell hyperplasia and the ratio

of old to regenerated cells is difficult to discern because of the severe vacuolation by fat. Liver cell necrosis is often present in the foci of acute inflammation (Fig. 16). Bile stasis is evident in many cases. Bile duct proliferation is usually absent, but biliary canaliculus proliferation is often present. The sinusoids and small central veins are inconspicuous, and the lobules appear relatively bloodless. Kupffer cells also are inconspicuous. The fat is abundant, its droplets are large, and it is present in all parts of the lobule; if any cells are spared, they are usually periportal. Nowhere is there an accumulation of portal tracts in the fibrous tissue as in postnecrotic cirrhosis.

Florid cirrhosis needs to be distinguished only from fatty liver on the one hand and the other fat-containing types of cirrhosis on the other; these are portal cirrhosis, types C and D. The late stages of fatty liver may have an increase in fibrous tissue, but it does not form distinct bands or scars, and there is no nodularity from hyperplasia or regeneration. No doubt, there is a point at which fatty liver merges with or goes over into florid cirrhosis.[15] In most collections the problem is rarely encountered; if it were a serious problem in a collection of cases, this fact would make the material interesting. By applying the criteria for florid cirrhosis as given, portal cirrhosis type C is easily differentiated, but the type D, because of its intralobular fibrous extensions, is more difficult. However, it lacks the tiny foci of necrosis and leukocytes, and it is usually multilobular or mixed. These two types merge, and the separation becomes arbitrary. In the materials on which this study is based, the problem of differentiation was rare. It is noteworthy that, as here defined and used, the concept of florid cirrhosis is slightly different from that of Popper, Szanto and Parthasarathy.[16]

Application of the Classification

In practical use, the cases having an obviously distinctive appearance, such as biliary, pigmentary, parasitic, and central cirrhosis, are readily recognized. Of the remainder, all those showing abnormal accumulations of portal tracts in the fibrous areas are put into the postnecrotic class. The examples of florid cirrhosis are recognized by the criteria already given. All of the remainder are examples of portal cirrhosis, and they are subclassified by the criteria given.

If the cirrhotic liver is obviously fatty, it falls into either the florid or the portal types C or D groups. The only exceptions to this rule are occasional cases of pigmentary cirrhosis having considerable amounts of fat, and, in some collections, a few cases of postnecrotic cirrhosis with fat. The exceptions to the simple rule are few in the collections of cases

studied to date, and if they were numerous, it should be suspected at once that the material is significantly different.

Most of the "wide band" forms of cirrhosis fall into the postnecrotic class and most of the "thin band" cases are portal, but many exceptions are found to these generalizations (Figs. 3 and 11).

This classification is applicable only to cases in which the microscopic sections adequately sample the nodules and the scars. The larger and more numerous the slides, the more accurate is the classification. Oversize slides are helpful in the beginning, especially in the coarsely nodular and irregularly scarred organs. The number and size of the sections can be reduced if the sampling is intelligently done from known places which are carefully described and recorded. The worst, best, and average areas are all sampled, including scars and nodules of all sizes. Special stains are useful but not essential; of them all, the silver reactions for reticulum are the most helpful, especially in the beginning. Needle biopsy specimens are generally not suitable even though they may be adequate for diagnosis.

Most of the diagnoses are made under low power magnification. A very low power scanning lens is desirable though not essential to cover the requisite numbers of sections adequately. It is constantly necessary in making interpretations to keep in mind the normal structures and to think in terms of the third dimension of the tissue, thus realizing that a marginal section along a portal tract is not a septum, that a marginal slice through a large nodule does not represent a small nodule, and that two adjacent portal tracts formed by a normal bifurcation do not mean juxtaposition by collapse.

Relatively distinct, objective groupings and sharp cut-off points are provided by this classification. The only points of merger are, firstly, between the postnecrotic and the portal B group, and, secondly, between florid cirrhosis and the portal D group. In practice, postnecrotic cirrhosis is diagnosed if the scars contain, at some point, 3 or more portal areas, and portal B cirrhosis if there are only 2. Portal type B is kept separate from the portal A group because it is sometimes difficult if not impossible, when only 2 triadal groups of structures are present, to determine whether they represent a point of bifurcation or a collapse. The florid cirrhosis group is separated from the portal D class on the basis of collections of neutrophils and narrow, poorly-formed, loose collagenous septums. Moreover, florid cirrhosis is nearly always monolobular whereas the portal D variety may be either monolobular, multilobular or mixed. It must also be conceded that an advanced biliary cirrhosis may come to resemble some of the portal types, but this event is so rare as to have no important influence on statistics in routine groups of cirrhosis cases.

COMMENTS

The purpose of this paper has been to describe a classification which can be used to characterize different lots of cirrhosis, both quantitatively and qualitatively, for statistical comparisons for studies in etiology and pathogenesis. Although a few comments have been made about the possible relationships between these types, this subject has been largely avoided in the interest of first establishing a usable base line from which to proceed.

Two methods for the formation of cirrhosis have long been recognized. The genesis of its fibrous scars from areas of necrosis and collapse have been conceded ever since it was known that sublethal cases of acute yellow atrophy and of toxic necrosis could go on to cirrhosis. On the other hand, the evolution of a periportal or a centrilobular fibrosis by accretion of additional fibrous tissue to cirrhosis of the obstructive biliary and the cardiac types is also not disputed. In a third and large group of cases, the pathogenesis is less clear. These include the examples, here called portal cirrhosis, in which slender fibrous bands, sometimes long and separating normal-appearing lobules, are found. These bands may by-pass many portal areas and separate lobules that appear normal. No type of long linear necrosis or hepatitis, which might produce these bands by collapse or by accretion of collagen, has ever been described. Only by assuming that there had been collapse, followed by the regeneration of large nodules—some of which, at least, contain many histologically normal lobules—can these long scars be explained on a postnecrotic basis. It is emphasized that collapse scars may result from complete atrophy as well as from necrosis.

Some of these thin long fibrous scars in the large-nodule cases must be bands or cords rather than membranes. If they were membranes, the section would, on the basis of chance, occasionally strike the membrane, even if it were thin, in such a plane as to show it to be a broad scar. In some instances this does not happen, even in a liberal sampling with a number of large sections. One can only conclude that the fibrous tracts in these cases are either bands or cords, even though in other instances they be membranes.[11]

Since the genesis of the scars in some cases of portal cirrhosis, as it is here defined, is equivocal, whereas it is beyond dispute in postnecrotic cirrhosis, it would seem to be desirable to separate these two types until the situation is clarified by further study. It is proposed that the criteria here described are adequate to draw an objective end point between the two groups. If further investigation shows that the separation is artificial and that both of these groups are postnecrotic, the division can then be abolished as arbitrary and undesirable.

If portal cirrhosis also results from necrosis and collapse, it must do so on the basis of collapse of less than entire lobules, because the scars do not contain approximated portal tracts. There are those who insist that there is only one pathogenesis for the main types of cirrhosis,[17] and it must be conceded that in terms of ultimate pathologic processes at the cellular level, this is a possibility.

The role of fat in the genesis of human cirrhosis is still in dispute. Some believe it plays no role in the pathogenesis of the human disease,[18] whereas others look upon it as contributing to the injury that results in the fibrous tissue increase.[15] Until this issue is settled, it seems desirable to separate the fatty types of portal cirrhosis (types C and D) from the fat-free form (type A). Probably most observers would agree that the fat in some cases is secondary, but reliable criteria by which fat of the secondary type and of the allegedly primary type may be recognized and distinguished have not been provided. Until the role of fat in the pathogenesis of cirrhosis is clarified, it seems desirable to put the livers containing fat into separate groups.

In a previous publication on cirrhosis in Africa,[5] the term Laennec cirrhosis was used. This term is now abandoned for this particular group in favor of the term "portal cirrhosis" because the original description by Laennec and the concept still prevailing in many French, Spanish, and Italian publications would accommodate only a part of the group as here defined, whereas "portal" adequately includes the entire group. In another publication on cirrhosis in Africa,[19] the present classification was abridged so that the term portal cirrhosis without fat was the present "type A" and portal cirrhosis with fat was equivalent to the present types C and D.

Summary

The main group of cirrhosis cases, remaining after the definitive special types such as pigmentary, biliary, central, parasitic, etc., have been removed, have been classified as postnecrotic, portal, and florid on microscopic bases which are illustrated. Postnecrotic cirrhosis is recognized by the stigmas of previous lobular collapse, identified chiefly by the concentration and juxtaposition in the fibrous scars of 3 or more portal tracts. Florid cirrhosis is characterized by distinctive criteria. Portal cirrhosis shows no evidence of previous lobular collapse, and it is subdivided into 4 classes. This classification agrees essentially with that established at the Havana and Kampala conferences, but provides objective, precise criteria for a sharp end point between postnecrotic and portal cirrhosis. It also adds florid cirrhosis and subdivides the portal group. The classification, tested by a comparison of 9 case col-

lections in Africa and one in the United States involving 1,355 cirrhotic livers, was sensitive enough to reveal some ethnic and geographic differences. As etiologic and pathogenetic knowledge on cirrhosis increases, these data based on structure can be reclassified and reinterpreted. The method is recommended for precise classification so that the cirrhosis in different case collections around the world can be accurately compared.

References

1. Report of the Board for Classification and Nomenclature of Cirrhosis of the Liver. Sherlock, S. (chairman). Fifth Pan-American Congress of Gastroenterology, La Habana, Cuba, January 20–27, 1956. *Gastroenterology,* 1956, **31,** 213–216.
2. Symposium in Kampala, August 1956. Cancer of the Liver Among African Negroes. *Acta Unio internat. contra cancrum,* 1957, **13,** 519–873.
3. Baggenstoss, A. H., and Stauffer, M. H. Posthepatitic and alcoholic cirrhosis: clinicopathologic study of 43 cases of each. *Gastroenterology,* 1952, **22,** 157–180.
4. Steiner, P. E. Cancer of the liver and cirrhosis in trans-Saharan Africa and in the United States of America. *Cancer,* 1960. (In press.)
5. Steiner, P. E., and Davies, J. N. P. Cirrhosis and primary liver carcinoma in Uganda Africans. *Brit. J. Cancer,* 1957, **11,** 523–534.
6. Steiner, P. E.; Camain, R., and Netik, J. Observations on cirrhosis and liver cancer at Dakar, French West Africa. *Cancer Res.,* 1959, **19,** 567–580.
7. Karsner, H. T. Morphology and pathogenesis of hepatic cirrhosis. *Am. J. Clin. Path.,* 1943, **13,** 569–606.
8. MacMahon, H. E. Biliary cirrhosis. Differential features of seven types. *A.M.A. Arch. Int. Med.,* 1958, **102,** 841–846.
9. Mallory, G. K., and Mallory, T. B. The Liver. In: Progress in Fundamental Medicine. McManus, J. F. A. (ed.). Lea & Febiger, Philadelphia, 1952, pp. 91–138.
10. Steiner, P. E. Nodular regenerative hyperplasia of the liver. *Am. J. Path.,* 1959, **35,** 943–953.
11. Popper, H., and Elias, H. Histogenesis of hepatic cirrhosis studied by the three-dimensional approach. *Am. J. Path.,* 1955, **31,** 405–441.
12. MacDonald, R. A., and Mallory, G. K. The natural history of postnecrotic cirrhosis. A study of 221 autopsy cases. *Am. J. Med.,* 1958, **24,** 334–357.
13. Higginson, J.; Grobbelaar, B. G., and Walker, A. R. P. Hepatic fibrosis and cirrhosis in man in relation to malnutrition. *Am. J. Path.,* 1957, **33,** 29–53.
14. Waterlow, J. C., and Bras, G. Nutritional liver damage in man. *Brit. M. Bull.,* 1957, **13,** 107–112.
15. Popper, H.; Szanto, P. B., and Elias, H. Transition of fatty liver into cirrhosis. *Gastroenterology,* 1955, **28,** 183–192.
16. Popper, H.; Szanto, P. B., and Parthasarathy, M. Florid cirrhosis. A review of 35 cases. *Am. J. Clin. Path.,* 1955, **25,** 889–901.
17. Dible, J. H. Degeneration, necrosis, and fibrosis in the liver. *Brit. M.J.,* 1951, **1,** 833–841.
18. Dible, J. H. Fatty change, cirrhosis and liver cancer in Great Britain. *Acta Unio internat. contra cancrum,* 1957, **13,** 545–552.

19. STEINER, P. E., and HIGGINSON, J. Definition and classification of cirrhosis of the liver. (In press.)

The author acknowledges with gratitude the use of photomicrographs made from material in the following collections: Prof. J. N. P. Davies, Makerere College Medical School, Kampala, Uganda, for Figure 1; Prof. R. Camain, Institut Pasteur, Dakar, French West Africa, for Figures 2 and 6 to 9; and Dr. J. Netik, Hôpital le Dantec, also of Dakar, for Figure 3; Prof. J. G. Thomson, Department of Pathology, University of Cape Town, Cape Town, South Africa, for Figures 13 and 15.

LEGENDS FOR FIGURES

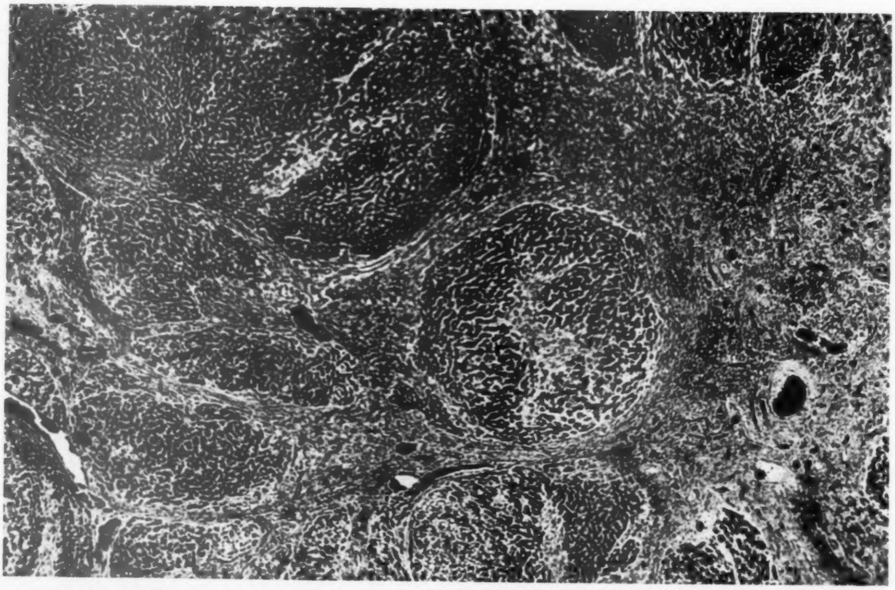
Except where indicated, photographs were prepared from sections stained with hematoxylin and eosin.

FIG. 1. Postnecrotic cirrhosis. A low power view of a severe (grade ++++), active (grade +++) cirrhosis of mixed nodule type. At the right is a large area of collapse-fibrosis containing the portal structures of many triads. Fibrous tracts separate nodules of liver parenchyma in which regenerated liver is distinguished from the original organ with difficulty because of the extensive secondary changes. White female, age 59 years. × 25.

FIG. 2. Postnecrotic cirrhosis. Higher magnification of a moderately severe (grade +++), moderative active (grade ++) cirrhosis, showing in the left lower corner an area of old collapse believed to contain 3 portal tracts. The hepatic parenchyma all appears to be of the regenerated type. Toucouleur male (true Negro) from the Senegal. Dakar, Institut Pasteur (no. 448A). × 75.

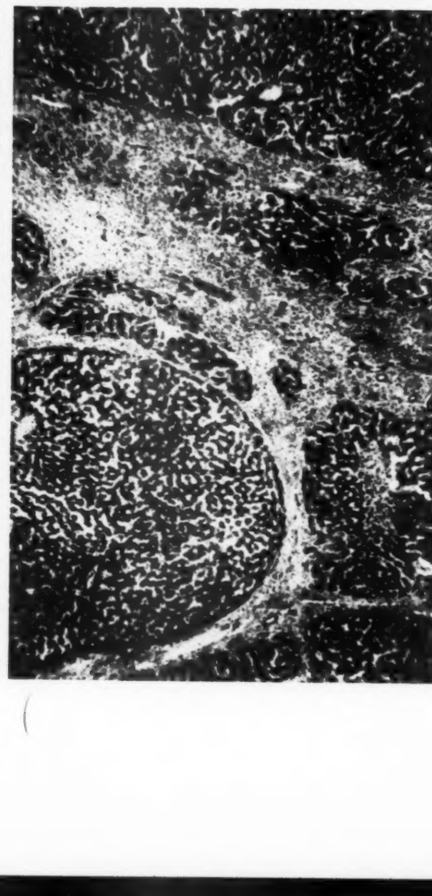
FIG. 3. Postnecrotic cirrhosis. This is not recognizable as postnecrotic cirrhosis from this area alone, no concentration of portal areas being apparent in the scars of this field. Moderately severe and active. The septums in the bottom half are slender. This case demonstrates the need for multiple sections. This liver also contained a primary carcinoma. Ouolof male, age 28 years, from Dakar, French West Africa. Hôpital le Dantec (no. 766). × 25.

2



1

2

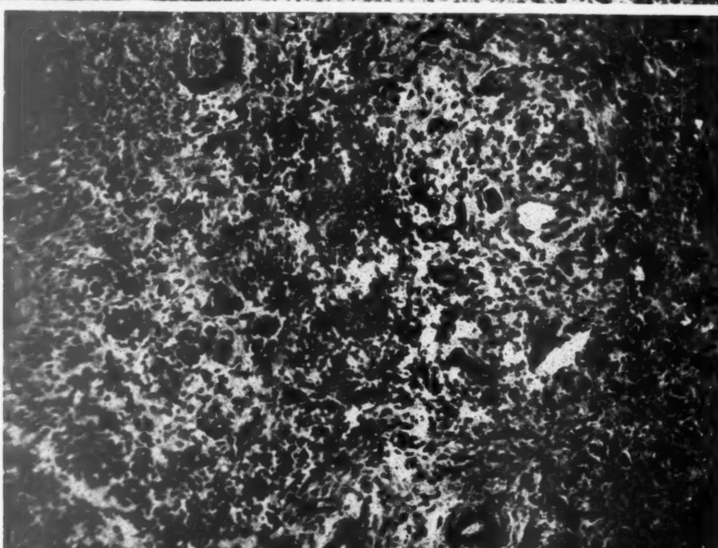


3

FIG. 4. Postnecrotic cirrhosis. The photograph shows much necrosis of entire nodules in a liver having also an older cirrhosis. The nodular character is apparent. From such areas, by further collapse, large scars are formed. From the surviving groups of liver cells, it is believed that regeneration may occur in favorable cases. A little proliferation of small bile ducts is apparent. This type presents no problem in diagnosis. Uganda (no. 166/55). × 42.

FIG. 5. Postnecrotic cirrhosis. High power view of an area of recent collapse, showing 3 distinct portal tracts, recognizable by their structures and the denser concentric connective tissue of the original tracts. There is also a considerable proliferation of cholangioles. Believed to be post-toxic (atophan). White female, age 75 years, in Chicago (no. 2893). × 85.

4



5

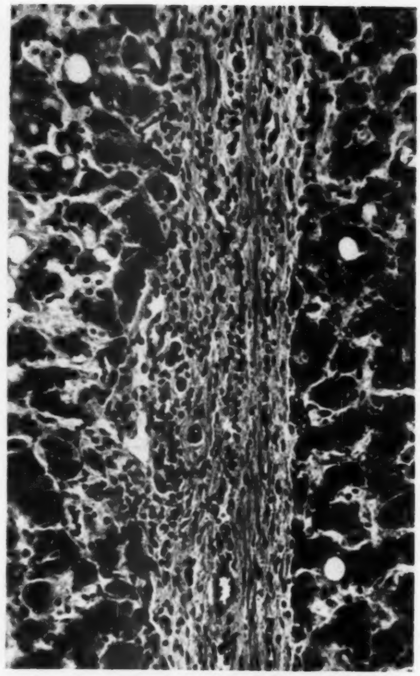
Fig. 6. Postnecrotic cirrhosis, showing a portion of an area of recent collapse in which 10 portal tracts were counted under the microscope, but because of the intense inflammatory reaction only 2 large tracts near the center are obvious in the photograph. In addition, some of the small bile ducts are original triadal ducts and others are new ducts, but the distinction cannot be recognized in the illustration. Senegalese. Dakar, Institut Pasteur (no. 1867A). × 75.

Fig. 7. Postnecrotic cirrhosis. A long and thick but only moderately active fibrous septum is manifest. Triadal structures are not evident here, so that the diagnosis of postnecrotic cirrhosis was based on areas other than this field. Typical cirrhosis from Uganda; Makerere (no. 413/52). × 135.

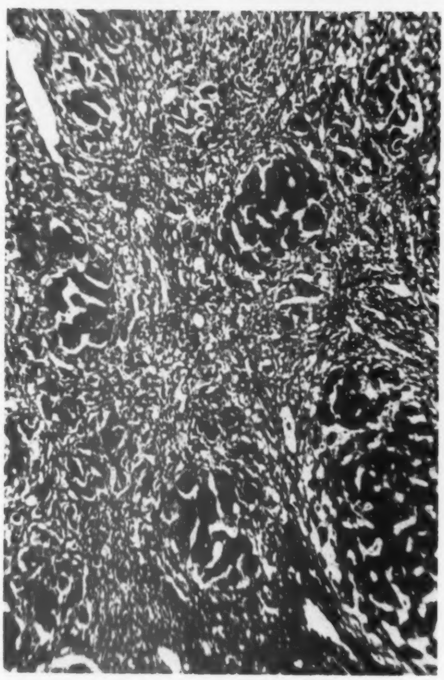
Fig. 8. Postnecrotic cirrhosis. A silver stain showing partial collapse of the hepatic stroma, except in persisting groups of hepatocytes. The larger spaces represent empty blood vessels. Ouolof female from Dakar; age 25 years, with 750 gm. liver. Institut Pasteur (no. 8355A). × 75.

Fig. 9. Postnecrotic cirrhosis. A silver reaction showing, across the center, a septum composed of condensed and partly fused stromal fibers and containing empty spaces, representing portal structures. Above and below this band are areas of pale, partly necrotic liver tissue not yet collapsed, although a little accretion to the septum is seen below. At the top is some dark, intact liver. Socé male, age 38 years, with liver weighing 1,200 gm. Dakar, Institut Pasteur (no. 6170A). × 75.

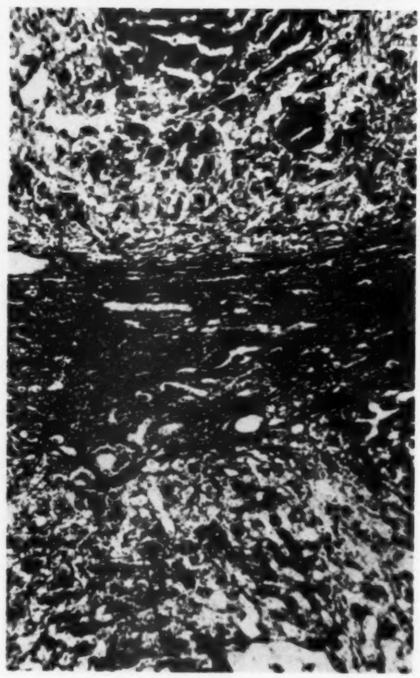
6



7



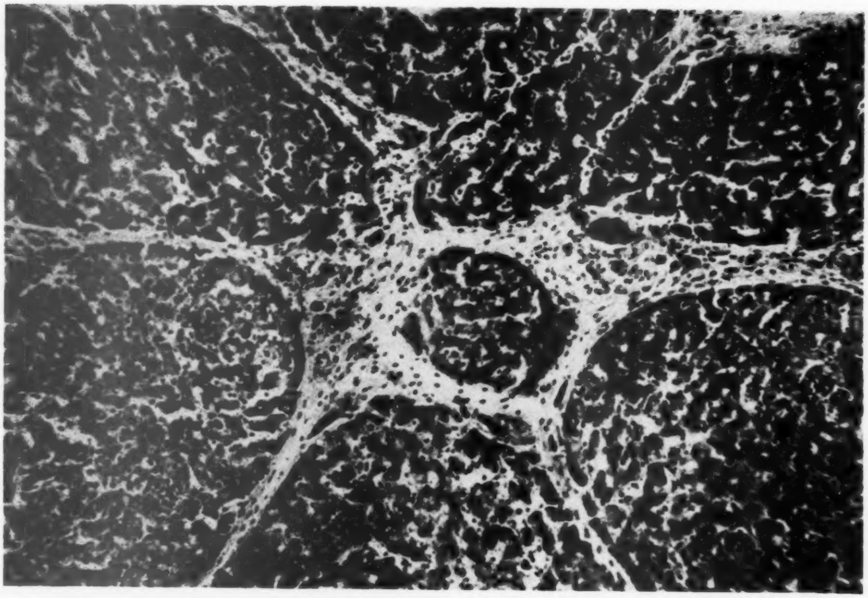
8



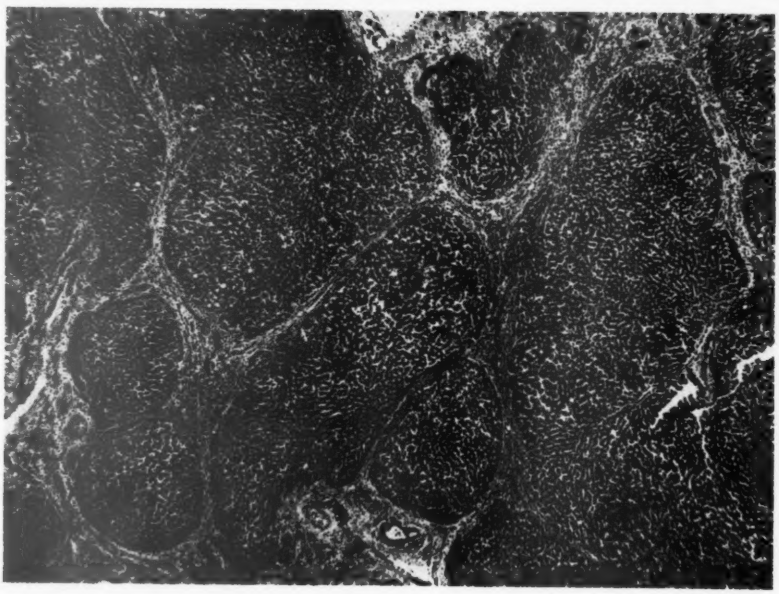
9

FIG. 10. Portal cirrhosis, type A. Typical case, showing nodules of liver tissue, some of which is new, separated by fibrous septums. This case would be ++ in severity, and + in the degree of activity. Chicago Negro man, age 47 years. Liver weight, 1,960 gm., uniformly slightly nodular. × 85.

FIG. 11. Portal cirrhosis, type A. The nodules are variable in size, as are the fibrous bands. At no place in the fibrous areas is there any concentration of portal tracts which would indicate collapse in the past. Severity is grade ++++ and activity is ++. White Chicago female, age 60 years. × 25.



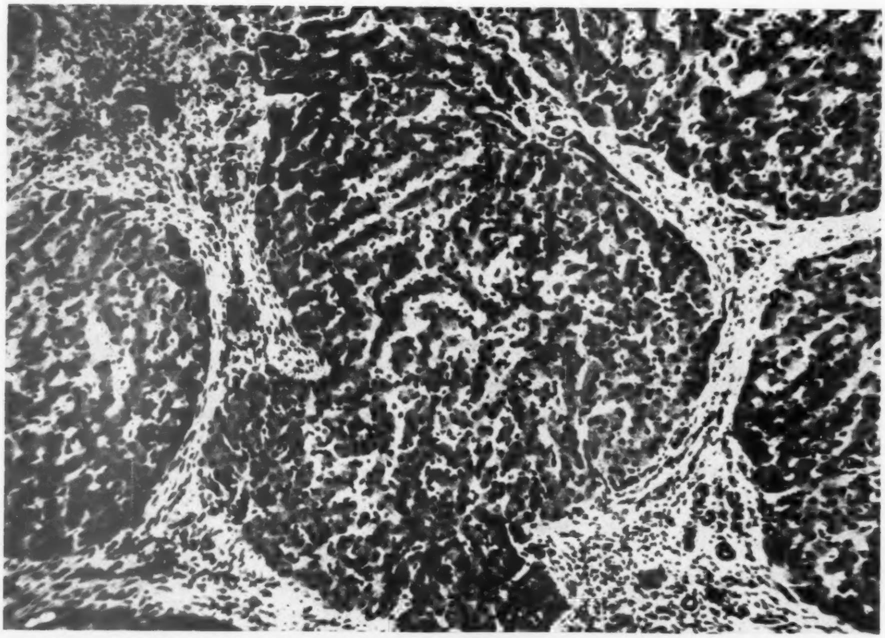
10



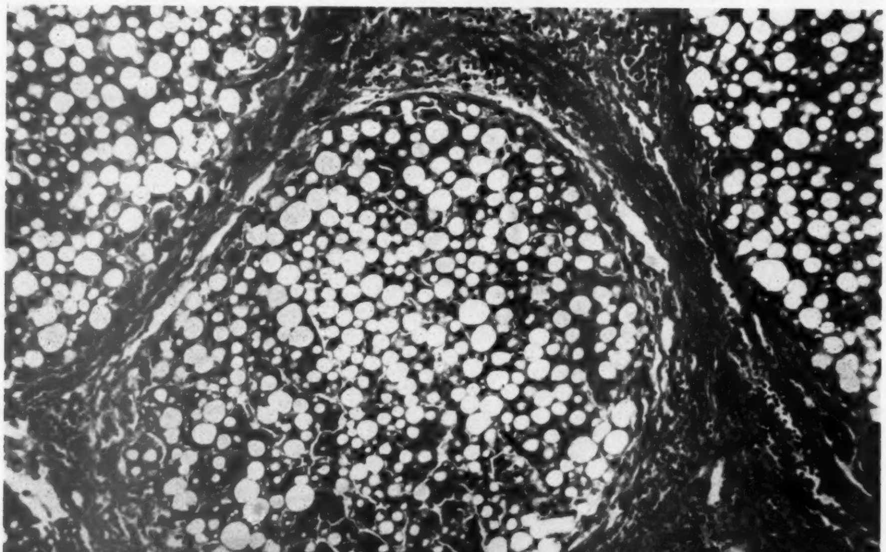
11

FIG. 12. Portal cirrhosis, type B. This resembles type A except that, in addition, there is a question whether 3 or more portal tracts are present in some scars. In this case the scar at the right lower corner was one of those in question. If it could have been shown that collapse of lobules had previously occurred, this case would have gone into the postnecrotic group. This area would be graded ++ in degree of activity and +++ in severity of the cirrhosis. White Chicago woman, age 65 years. Liver uniformly nodular and 1,265 gm. × 80.

FIG. 13. Portal cirrhosis, type C. Resembles type A except that it has, in addition, much fat of the large droplet type. White male, age 76 years, with history of alcoholism. Liver nodular, weight 2,505 gm. University of Cape Town (no. 278/53). × 85.



12



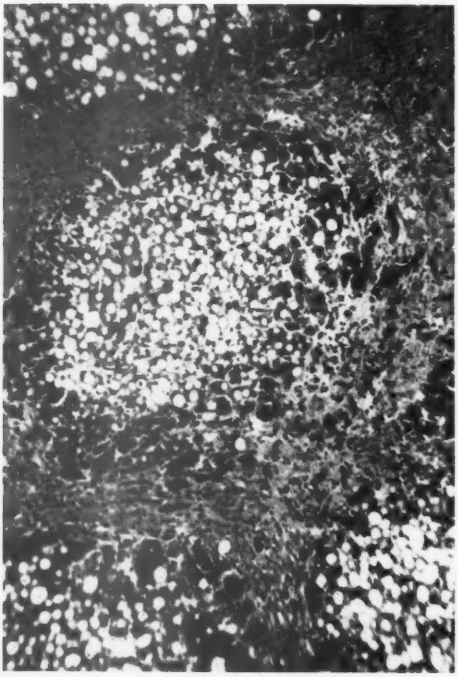
13

FIG. 14. Portal cirrhosis, type D. Resembles type C but has, in addition to the fat, extensions of delicate fibrous tissue from the main septums into the nodules. White female, Chicago, age 54 years; liver weight 1,290 gm. × 75.

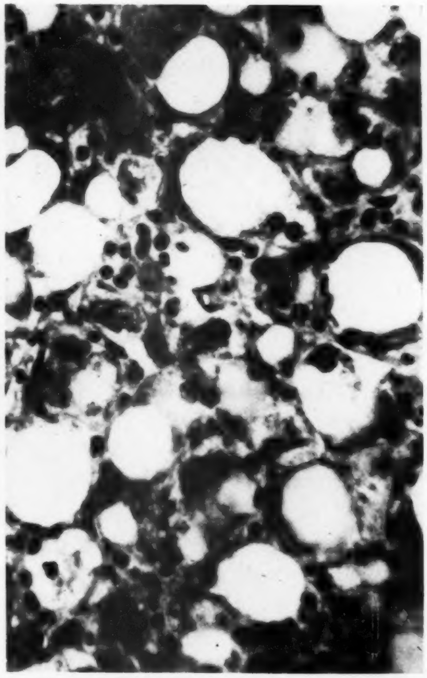
FIG. 15. Florid cirrhosis. Shows fibrous tissue increase, which is becoming linear and septal in some places, and nodular regeneration. Besides an inflammatory reaction, which includes neutrophils, there is fat and bile. The nodule at the upper right, which appears to be regenerated liver, is free of fat. White male, age 32 years, ill only 5 weeks; history of alcoholism. Liver weight 2,965 gm. University of Cape Town (no. 29/56). × 110.

FIG. 16. Florid cirrhosis. A high power magnification to show the inflammatory reaction with neutrophils and the severe degenerative cytoplasmic changes, apart from the fat. White Chicago male, age 52 years, with history of alcoholic abuse. Liver weight 3,700 gm. × 322.

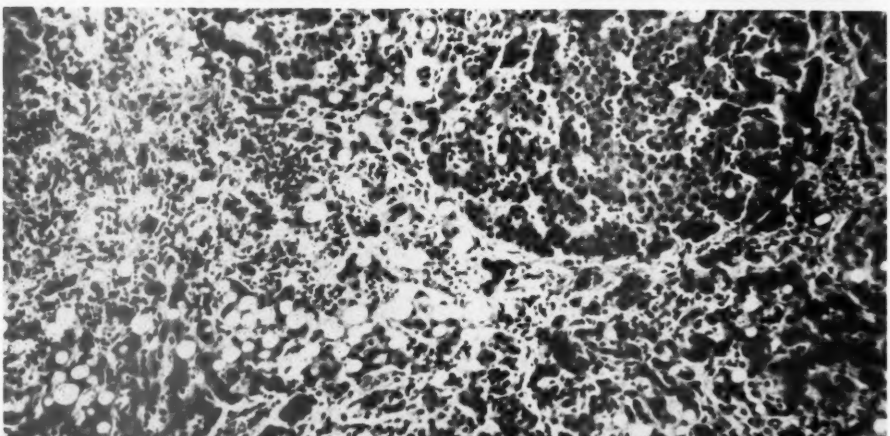
14



14



15



16

"ALCOHOLIC" HYALIN IN HUMAN CIRRHOSIS HISTOCHEMICAL STUDIES

Stanlislav A. Norkin, M.D., Raymond Weitzel, M.D., Dante Campagna-Pinto, M.D., Richard A. MacDonald, M.D. and G. Kenneth Mallory, M.D.

From the Mallory Institute of Pathology, Boston City Hospital, and the Departments of Pathology of Boston University School of Medicine and Harvard Medical School, Boston, Mass.

In 1901 Mallory[1,2] described peculiar aggregates of cytoplasmic material which he termed "alcoholic hyalin" in the hepatic cells of patients with fatty liver and cirrhosis. He ascribed this alteration to degenerative changes of liver cells, with the appearance, first, of minute hyaline droplets in the cytoplasm, followed by fusion of the droplets to form an irregular intracytoplasmic meshwork located about the nuclei of affected cells. With rare exceptions these changes have in this laboratory been found to be confined to the livers of patients with a history of alcoholism, although the extent and the nature of this relationship has not been clarified. In the present investigation, histochemical procedures were carried out on livers containing alcoholic hyalin in an attempt to obtain information concerning its nature and composition.

Relatively little work has been done on the staining characteristics of alcoholic hyalin. In the usual hematoxylin and eosin (H and E) staining, it is poorly stained as an eosinophilic, slightly refractile portion of the cytoplasm. With phloxine methylene blue (PMB) staining after Zenker's acetic acid fixation, Mallory[3] noted that hyalin stained a prominent red and was readily identified as a discrete, ropelike, eosinophilic, refractile component of liver cells, with a characteristic perinuclear location. Alcoholic hyalin is to be distinguished from other types of hyaline material in liver cells, such as nonspecific droplets, or "hyaline bodies" or "Councilman-like bodies" in viral hepatitis.[4] Pappenheimer and Hawthorne,[5] for example, encountered intracytoplasmic hyaline bodies in human livers in many different types of hepatic disease. They considered these to be examples of "alcoholic hyalin." Examination of the published photomicrographs, however, indicates that they were not dealing with alcoholic hyalin as described by Mallory. In 1949 Edwards and Mallory[6] applied the phosphotungstic acid-hematoxylin (PTAH) stain to livers with alcoholic hyalin after fixation in 95 per cent or abso-

Supported by a contract with the Office of the Surgeon General, Department of the Army (DA-49-007-MD-863) and aided in part by grants from the United States Public Health Service (C-4119 and C-4666).

Received for publication, October 15, 1959.

lute ethyl alcohol. They reported a differential demonstration of the hyaline meshwork which appeared as a purple, perinuclear cellular component. Laqueur [7] in 1950 observed selective staining of alcoholic hyalin by acid fuchsin; with this stain, hyalin resisted controlled decolorization procedures and stained a deep red. In 1950, McKay [8] reported that basophilic cytoplasmic bodies usually related to protein synthesis and associated with cytoplasmic ribonucleic acid were absent from alcoholic hyalin. Roque in 1955 [9] reported a selective stain for alcoholic hyalin, using aniline blue and chromotrope 2R at an acid pH. On the basis of enzymatic digestion as well as staining procedures, he postulated that the hyalin was a conjugated protein, a basic protein containing significant amounts of arginine, with maximal binding of acid dyes at pH 1.8 to 2.2. He suggested that the nonprotein prosthetic group was a carbohydrate material. He noted that some alcoholic hyalin, usually with a fine droplet appearance, showed an affinity for the aniline blue component of the aniline blue-chromotrope 2R stain. He termed this "young" hyalin and ascribed the staining affinity to the presence of glycoprotein not present in the "old," or more coarse, hyalin. The latter usually stained with the chromotrope 2R portion of the stain.

Material and Methods

Hepatic tissue obtained from 74 cirrhotic patients was used in this investigation. In 60 cases the liver was obtained at necropsy, and in 14 patients, tissue was obtained by Vim-Silverman needle biopsy or by surgical biopsy at the time of laparotomy. Necropsy tissues were obtained 2 to 14 hours after death, with an average of 8 hours; the biopsy specimens were fixed immediately upon removal from the body. All specimens were examined for the presence of alcoholic hyalin by conventional methods before special procedures were performed. Tissues obtained at necropsy were fixed in 10 per cent neutral buffered formalin,[10] in Zenker's solution containing 5 per cent glacial acetic acid,[10] in 10 per cent neutral buffered formalin containing 1 per cent calcium chloride, in absolute ethyl, methyl and isopropyl alcohol, and in cold (0° to 4° C.) acetone. Tissues were also "quick frozen" with iso-pentane cooled with liquid nitrogen, followed by freeze-dry substitution [11] or were frozen-dried,[11] and embedded in paraffin and in Carbowaxes. Biopsy tissues were fixed by formalin, Zenker's or alcohol solution, and additional portions were frozen in iso-pentane.

In addition to several histologic stains, histochemical procedures were employed. These are set forth in detail below. Throughout this paper the terms "fine" and "coarse" are used to separate two types of hyalin, according to its appearance in phloxine methylene blue stained sections.

Results

General Histologic Methods

In unfixed frozen sections examined by phase contrast microscopy, alcoholic hyalin was visible as an intracytoplasmic refractile network, its appearance similar to that in stained sections. The appearance of hyalin when stained with hematoxylin and eosin and phloxine methylene blue has been described above. The method of fixation resulted in only slight differences; in general, Zenker's acetic acid fixation followed by phloxine methylene blue showed the hyalin best. The phosphotungstic acid-hematoxylin stain worked well on either alcohol or neutral formalin-fixed tissue. With frozen-dried tissue, aniline blue [10] or the chromotrope 2R stain [9] demonstrated hyalin well, as a purple aggregate of intracytoplasmic globules with the former stain, and as either a blue aggregate or a red aggregate with a blue rim with the latter (Fig. 1). With acid fuchsin stains [5,7] hyalin retained the acid fuchsin during decolorization and appeared as bright red aggregates. Regardless of the fixation methods employed, alcoholic hyalin was in all cases demonstrable by the above histologic stains.

Histochemical Procedures

With Congo red stain [11] hyalin retained the dye and stained orange red. With the phosphate stain of Serra and Querioz-Lopes,[10] it stained blue, but with the phosphate method of Serra and Feigl [10] it did not develop a sufficient color reaction. Acid fast stains [10] gave variable, usually negative results, with occasional retention of some color in the central portions of coarse hyalin. Hyalin was not stained by the benzidine stain for hemoglobin,[11] or by stains for hemosiderin,[10,11] oil red O and Sudan black B stains in paraffin sections for ceroid,[11,12] osmic acid,[10] iodine stains for bile pigments,[11] or Gomori's paraldehyde fuchsin stain.[13]

Techniques designed for demonstration of alkaline phosphatase,[11] acid phosphatase,[14] lipase,[15] esterases,[10,16-18] 5-nucleotidase,[11] and succinic dehydrogenase,[10] performed on frozen-dried, fresh frozen, or acetone-fixed tissues were uniformly negative.

Further histochemical procedures were concerned with the staining of protein, carbohydrate and lipid. These are summarized in Tables I to III.

In addition to the staining reactions summarized in the tables, various solvent procedures were carried out. Frozen and frozen-dried tissues were treated with cold (0 to 4°) and hot (56° C.) acetone, hot pyridine (60° C.), hot ether (34° C.) and a mixture of hot chloroform with

Table I

ALCOHOLIC HYALIN
"PROTEIN" STAINING REACTIONS

Procedure	Reference no.	Type of hyalin: Fine	Type of hyalin: Coarse	Result
Feulgen	11	X*	X	Does not stain
Pyronin-methyl green	11	X	X	Stains light red
Alloxan, ninhydrin-Schiff (amino linkage)	30	X	X	Pink to red
Coupled tetrazonium alone †	11, 20, 24	X	X	Purple-brown
Following dinitrofluoro-benzene (DNFB) treatment †	11, 20, 24	X		Staining partially abolished
Following dinitrofluoro-benzene (DNFB) treatment	11, 20, 24		X	Staining completely abolished
Staining following benzoylation				
With heat (70° C., 10 min.)	11, 20, 24	X	X	Does not stain
Without heat	11, 20, 24	X	X	Does not stain
Performic acid treatment followed by tetrazonium stain	11, 20, 24	X	X	No change due to performic acid
DNFB for tyrosine †	11, 20, 24	X	X	Red to purple
Dimethylamino-benzaldehyde-nitrate †	11, 20, 24	X	X	Does not stain
Protein-bound SH groups (Barrnett-Seligman)	11, 20, 24	X		Red
Protein-bound SH groups	11, 20, 24		X	Red-purple
Sakaguchi reaction (modification) †	10, 11	X	X	Red or orange
Solubility studies followed by staining †	11, 23			
Buffers, pH 0.7–10.5	11, 23	X	X	No change in staining with phloxine methylene blue, aniline blue or coupled tetrazonium reactions
HCl, 0.1, 0.5, 1.0 N	11, 23	X	X	
NaCl, 0.1, 0.3, 1.0 N	11, 23	X	X	
Urea, 1, 5, 10%	11, 23	X	X	
Staining with various dyes in buffers of pH 0.7–9.25 †	9, 22	X	X	Binds acid dyes, with maximum affinity at pH 2.0
Enzymatic digestion followed by staining	11			
Pepsin, 7–10 min.	11	X	X	Staining accentuated
Pepsin, 20+ min.	11	X	X	Hyalin disappears
Trypsin < 1 hr.	11	X		Hyalin disappears
Trypsin, 1 hr.	11		X	Hyalin persists
Trypsin > 2 hr.	11	X	X	Hyalin disappears
Ribonuclease < 2 hr.	11	X	X	Hyalin persists. Note: basophilic bodies of liver cytoplasm disappear
Hyaluronidase, 3 hr.	11	X	X	Hyalin persists

* X indicates the type of hyalin upon which staining or other procedure was carried out.
† Preparation of tissues was by freeze-dry alone or in addition to other methods.

TABLE II

ALCOHOLIC HYALIN

"CARBOHYDRATE" STAINING REACTIONS

Procedure	Reference no.	Type of hyalin: Fine	Type of hyalin: Coarse	Result
Periodic acid-Schiff	10	X	X	Does not stain
Periodic acid-Schiff (Hotchkiss)	11	X	X	Stains yellow-orange with orange G fraction
Toluidine blue	11	X	X	Stains orthochromatically
Crystal violet	11	X	X	Stains orthochromatically
Alcian blue	11	X	X	Does not stain
Carmine (Best)	11	X	X	Does not stain
Methenamine silver reduction	10	X	X	Does not stain

TABLE III

ALCOHOLIC HYALIN

"LIPID" STAINING REACTIONS

Procedure	Reference no.	Type of hyalin: Fine	Type of hyalin: Coarse	Result
Oil red O * †	11	X	X	Does not stain
Sudan black B in propylene * †	11	X	X	Does not stain
Sudan III * †	11	X	X	Does not stain
Acid hematin	11	X		Blue
Acid hematin	11		X	Darker blue
Acid hematin, pyridine extraction	11	X	X	Staining not affected
Myelin stain (Lillie) * †	10	X	X	Black
Myelin stain (Loyez) ‡	10	X	X	Black
Luxol fast blue * †	29	X	X	Dark blue

* Fixation was in 10% neutral buffered formalin containing 1% CaCl; stains were performed on both Carbowax and paraffin embedded tissue.

† In addition, other fixatives and freeze-dried preparations were used.

‡ Celloidin embedded.

methyl alcohol. After 24 hours, sections were stained with aniline blue-acid fuchsin [19] and Lillie's myelin stain.[10] There were no changes in the staining characteristics of hyalin due to the solvent procedures. This appeared to indicate that the phospholipid staining procedures were not specific.[20] Frozen-dried tissues were heated with 2 and 5 per cent solutions of deoxycholate at 37° C. for 48 hours and were incubated with a culture of *Clostridium welchii.* The only alteration in the staining features of hyalin was that it lost its ability to retain the acid fuchsin portion of the aniline blue-acid fuchsin stain. Frozen-dried sections were acetylated with cold (0 to 4° C.) acetic anhydride for 48 hours, and were deaminated by van Slyke's procedure [10] using nitrous acid at 22 ° C.

for 6 to 8 hours. Following both procedures, there was abolition of the aniline blue staining.

Discussion

The detection, by means of phase contrast microscopy, of alcoholic hyalin in unfixed, frozen sections, and its presence in tissues fixed in a variety of ways and with a variety of stains indicate that it is a true morphologic entity and not an artifact of fixation. Negative stains for benzidine show that hyalin does not contain hemoglobin or red blood cells; negative oil red O and Sudan black B stains after paraffin embedding indicate that it is not related to ceroid.[12] Negative lipid stains in Carbowax-embedded material, in spite of an association with fatty, nutritional, or "alcoholic" cirrhosis, indicate that the hyalin is not a lipid. Similarly, carbohydrate stains were negative. Enzyme techniques and deoxyribonucleic acid (DNA) procedures were also negative; methyl green-pryonin stains were usually weakly positive or negative, showing that ribonucleic acid (RNA), a normal cytoplasmic component, was present only occasionally. Hyalin did not contain bilirubin (negative iodine stains) in spite of the frequent but not invariable association with hepatic decompensation and bile stasis.

The occurrence of alcoholic hyalin in two forms, as a fine meshwork or an aggregate of eosinophilic droplets, and as a coarse aggregation of larger, more compact droplets, has been noted previously,[6,9] and may be a function of the time or severity. Hyalin may form first as fine droplets, later coalescing to form larger, more coarse aggregates. If this is the explanation, hyalin might be the result of a denaturation of cytoplasmic protein in viable hepatic cells. Parenchymal elements undergoing normal mitosis have been observed to contain alcoholic hyalin. In the present investigation, the protein staining reactions of hyalin, its resistance to solvents and extraction at varying levels of pH, and the initial resistance, followed by susceptibility to prolonged peptic and tryptic digestion, offer some support for the concept that it is a denatured protein.

Hyalin in conventional histologic preparations stains with acid dyes, suggesting that it is probably of basic nature.[21] This is supported by the observation that with controlled pH conditions, the hyalin stains most intensely at a pH of about 2.0, decreasing as the pH is raised. The influence of pH on staining may be explained by the amphoteric character of protein molecules and the effect of the pH of the medium on the degree and charge of dissociation of protein, in this case of basic groups.[21,22] The "free" basic groups that might be responsible for the reaction are the guanidine group (arginine, the ϵ amino group of lysine and the iminazole groups of histidine).[23] Arginine is demonstrable by specific

staining procedures, and is present in large quantities, as noted by Roque.[9] Specific stains for lysine and histidine are not available, and the presence of histidine can only be determined indirectly by the reactions given below.

The strong coupled tetrazonium reaction implies the presence of one or more of the amino acids, histidine, tyrosine or tryptophan[11,20,24] (Fig. 2). The dinitrofluorobenzene procedure, followed by the coupled tetrazonium reaction, completely abolishes the staining of coarse hyalin and markedly reduces the reaction of the fine hyalin, probably specifying tyrosine as the principal amino acid responsible for the tetrazonium reaction (Fig. 3). Oxidizing or "blocking" reactions for tryptophan with performic acid carried out prior to tetrazonium staining do not alter the results, and this, together with failure to demonstrate tryptophan by the Ehrlich nitrate reaction, makes it unlikely that tryptophan is responsible for the tetrazonium reaction.

The coupled tetrazonium reaction is completely negative following benzoylation, with and without heat pretreatment; this excludes the participation of purine and pyrimidine bases, according to Danielli.[20,24] The presence of sulfur-containing amino acids can be demonstrated by the Barrnett-Seligman procedure[25] for free sulfhydryl groups. In this procedure also, as in the case of tyrosine, the central portion of coarse hyalin reacts more strongly than outer portions, indicating a larger number of free SH groups.

Numerous procedures[11,23] show that the protein in hyalin is in insoluble form. These are: failure of extraction of hyalin or any noticeable change in its staining characteristics following exposure to water, buffer solutions in a range of pH, dilute salt solutions, alkalis, acids, and alcohols. The insolubility may be accounted for by changes in the protein molecule accompanying a process of denaturation or a protein-protein complex formation such as protamine or histone-acid protein complex.[23] Consistent with a rearrangement of protein molecules are the following observations: (a) the presence of two types of hyalin, coarse and fine forms; (b) an increase in reactive tyrosine and SH groups in the more coarse hyalin, particularly in its central portion; (c) an increase in reducing groups[20] as demonstrated by the alkaline tetrazolium method, which is probably due in large part to the presence of SH groups. The latter two alterations, liberation of free SH groups and unmasking of the phenolic group of tyrosine, usually accompany the process of denaturation.[23]

Staining with phosphomolybdic acid-aniline blue demonstrates hyalin, the staining disappearing if pretreatment by deamination procedures and mild acetylation are carried out. This probably indicates the presence

in hyalin of basic proteins, histones or protamines, in which free NH_2 groups give this sequence of reaction.[10] The inability to extract hyalin by protein solvents precludes the usual classifications of hyalin according to solubility. On the other hand, the presence of large numbers of reactive groups of arginine, free NH_2 groups, and of lysine, suggests a histone component.[23] Whether the more acid central portion[9] of coarse hyalin is the site of protein-protein complexes, which histones are known to form,[23] or a change in the number of reactive groups accompanying denaturation is not known. Although other groups such as phenolic, iminazole, SH and guanidine may participate in the reaction with nitrous acid,[10,23] the presence of the reactive free NH_2 groups indicates the presence of lysine as one of the amino acids of the protein fraction.

Stains to detect a prosthetic group, such as would be present if hyalin were a conjugated protein, were uniformly negative. In this respect, our results differ from those of Roque[9] who attributed the affinity of "young" alcoholic hyalin for aniline blue dye to the existence of a glycoprotein complex. In the present investigation, stains for phosphates gave positive reactions; this has been noted by others.[3] The meaning of these results is far from clear. Gomori[10,27] pointed out that although the chemical reaction is specific, the sites of reaction may be altered because of diffusion of a colored product and adsorption to protein.

Positive phospholipid stains may have been nonspecific and the result of physical retention of dye, rather than due to the presence of phospholipid in hyalin, since the reaction between the substance and the dye may depend on structural affinity[21] (Fig. 4). Negative Sudan black B stains would seem to preclude the existence of phospholipid in hyalin,[11,28] although a phospholipid fraction tightly bound to protein cannot be excluded with certainty by these methods. Extraction with pyridine before staining did not alter the results, however, with the Baker hematein stain; this is further evidence that the reaction was not due to phospholipid.[11] Similarly, there was an absence of noticeable change in staining following extraction with phospholipid and fat solvents.

Summary and Conclusions

A histochemical study was made of intracytoplasmic "alcoholic" hyalin in the livers of patients with fatty, nutritional (alcoholic) cirrhosis. Alcoholic hyalin appeared to be an insoluble basic protein complex or a denatured protein present within otherwise viable liver cells although the possibility that it may be a phospholipid protein complex cannot be ruled out.

References

1. Mallory, F. B. Necroses of liver. *J. Med. Res.*, 1901, **6**, 264–279.
2. Mallory, F. B. Cirrhosis of the liver. *New England J. Med.*, 1932, **206**, 1231–1239.
3. Mallory, F. B. Phosphorus and alcoholic cirrhosis. *Am. J. Path.*, 1933, **9**, 557–568.
4. Popper, H., and Schaffner, F. Liver: Structure and Function. The Blakiston Div., McGraw-Hill Book Co., New York, 1957, 777 pp.
5. Pappenheimer, A. M., and Hawthorne, J. J. Certain cytoplasmic inclusions of liver cells. *Am. J. Path.*, 1936, **12**, 625–634.
6. Edwards, J. E., and Mallory, G. K. Mallory's phosphotungstic acid hematoxylin stain of alcoholic hyalin. *Bull. Internat. A. M. Mus.*, 1949, **30**, 130–134.
7. Laqueur, G. L. A selective stain for cytoplasmic hyaline bodies in Laennec's cirrhosis. *Am. J. Clin. Path.*, 1950, **20**, 689–690.
8. McKay, D., and Farrar, J. T. Basophilic substances in human liver cells. *Cancer*, 1950, **3**, 106–115.
9. Roque, A. L. Chromotrope aniline blue method of staining Mallory bodies of Laennec's cirrhosis. *Lab. Invest.*, 1953, **2**, 15–21.
10. Lillie, R. D. Histopathologic Technic and Practical Histochemistry. The Blakiston Co., Inc., New York, 1954, 501 pp.
11. Pearse, A. G. E. Histochemistry, Theoretical and Applied. Little, Brown & Co., Boston, 1953, 503 pp.
12. Lee, C. S. Histochemical studies of the ceroid pigment of rats and mice and its relation to necrosis. *J. Nat. Cancer Inst.*, 1950–1951, **11**, 339–349.
13. Gomori, G. Aldehyde-fuchsin: a new stain for elastic tissue. *Am. J. Clin. Path.*, 1950, **20**, 665–666.
14. Gomori, G. An improved histochemical technic for acid phosphatase. *Stain Technol.*, 1950, **25**, 81–85.
15. Gomori, G. The microtechnical demonstration of sites of lipase activity. *Proc. Soc. Exper. Biol. & Med.*, 1945, **58**, 362–364.
16. Nachlas, M. N., and Seligman, A. M. The histochemical demonstration of esterase. *J. Nat. Cancer Inst.*, 1948–1949, **9**, 415–425.
17. Gomori, G. The Histochemistry of Esterases. In: International Review of Cytology. Bourne, G. H., and Danielli, J. F. (eds.) Academic Press, Inc., New York, 1952, Vol. 1, pp. 323–335.
18. Pearse, A. G. The intracellular localisation of esterases. (Abstract) *J. Path. & Bact.*, 1953, **66**, 331–333.
19. Mallory, F. B., and Wright, J. H. Pathological Technique. A Practical Manual for Workers in Pathological Histology and Bacteriology. W. B. Saunders, New York, 1924, ed. 8, 666 pp.
20. Danielli, J. F. A Study of Techniques for the Cytochemical Demonstration of Nucleic Acids and Some Components of Proteins. In: Symposia of the Society for Experimental Biology, Vol. 1, Nucleic Acids. University Press, Cambridge, 1947, pp. 101–113.
21. Singer, M. Factors Which Control the Staining of Tissue Sections with Acid and Basic Dyes. In: International Review of Cytology. Bourne, G. H., and Danielli, J. F. (eds.) Academic Press, Inc., New York, 1952, Vol. 1, pp. 211–255.

22. Fraenkel-Conrat, H., and Cooper, M. The use of dyes for the determination of acid and basic groups in proteins. *J. Biol. Chem.*, 1944, **154**, 239–246.

23. Greenberg, D. M. (ed.) Amino Acids and Proteins. Theory, Methods, Application. Charles C Thomas, Springfield, Ill., 1951, 950 pp.

24. Danielli, J. F. Studies on the cytochemistry of proteins. *Cold Spring Harbor Symp. Quant. Biol.*, 1950, **14**, 32–39.

25. Barrnett, R. J., and Seligman, A. M. Histochemical demonstration of protein-bound sulfhydryl groups. *Science*, 1952, **116**, 323–327.

26. Pearse, A. G. E. Application of the alkaline tetrazolium reaction to the study of reducing groups in tissue sections. *J. Path. & Bact.*, 1954, **67**, 129–136.

27. Gomori, G. Microscopic Histochemistry: Principles and Practice. University of Chicago Press, Chicago, 1952, 273 pp.

28. Palay, S. L. (ed.) Frontiers in Cytology. Yale University Press, New Haven, 1958, 529 pp.

29. Pearse, A. G. E. Copper phthalocyanins as phospholipid stains. *J. Path. & Bact.*, 1955, **70**, 554–557.

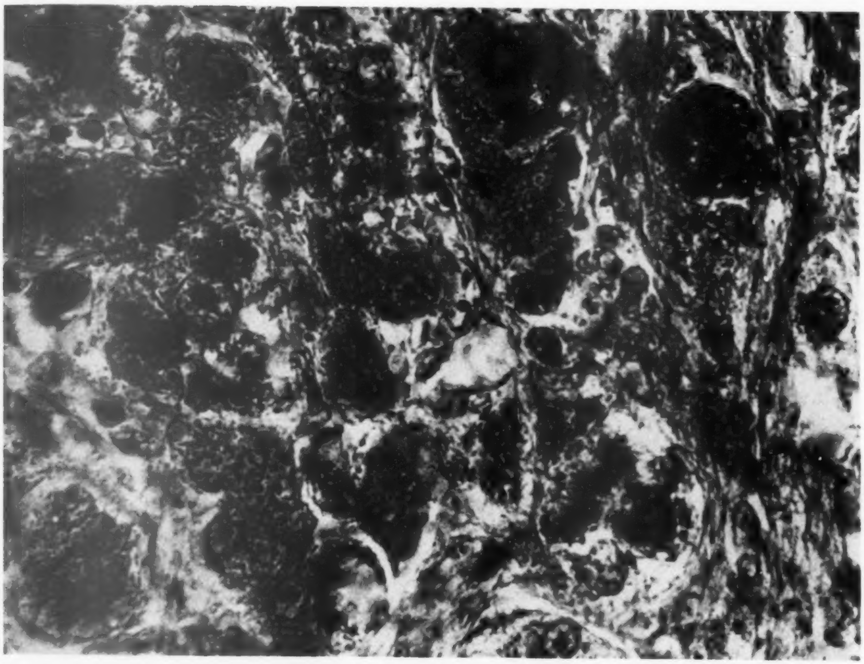
30. Yasuma, A., and Ichikawa, T. Ninhydrin Schiff and alloxan-Schiff staining. A new histochemical staining method for protein. *J. Lab. & Clin. Med.*, 1953, **41**, 296–299.

The generous cooperation and advice of Dr. Charles S. Davidson is gratefully acknowledged.

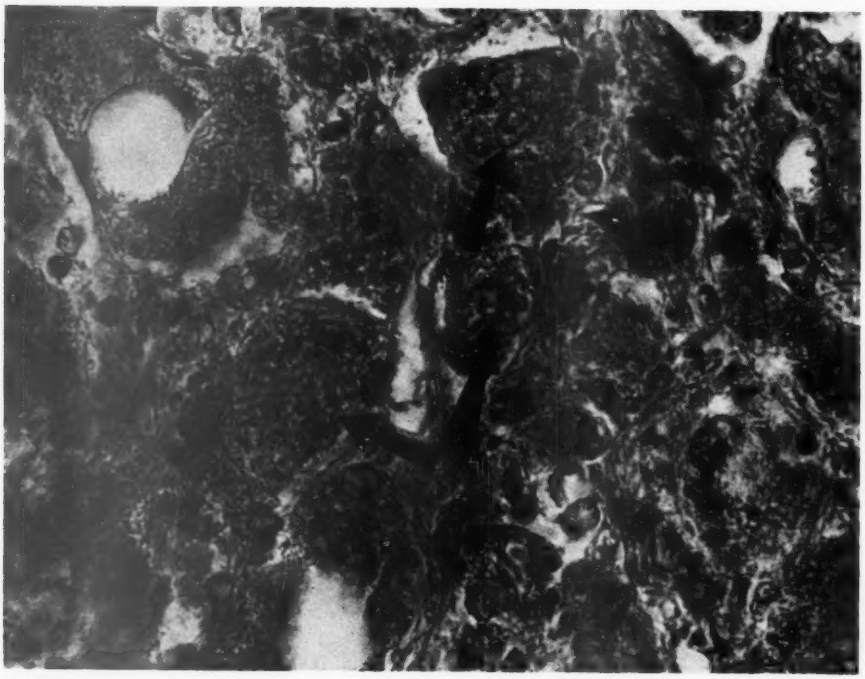
Legends for Figures

Fig. 1. Section of frozen-dried liver stained by the coupled tetrazonium method. The hyalin is prominent. × 700.

Fig. 2. The same block as that in Figure 1. Section pretreated with dinitrofluorobenzene, followed by the coupled tetrazonium stain. Arrows indicate areas of alcoholic hyalin, which do not stain. × 700.



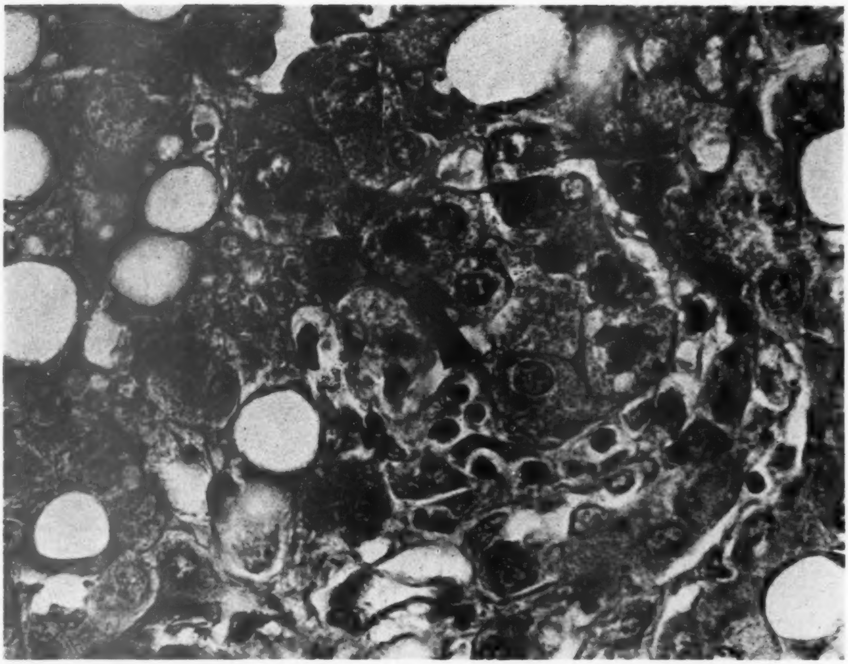
1



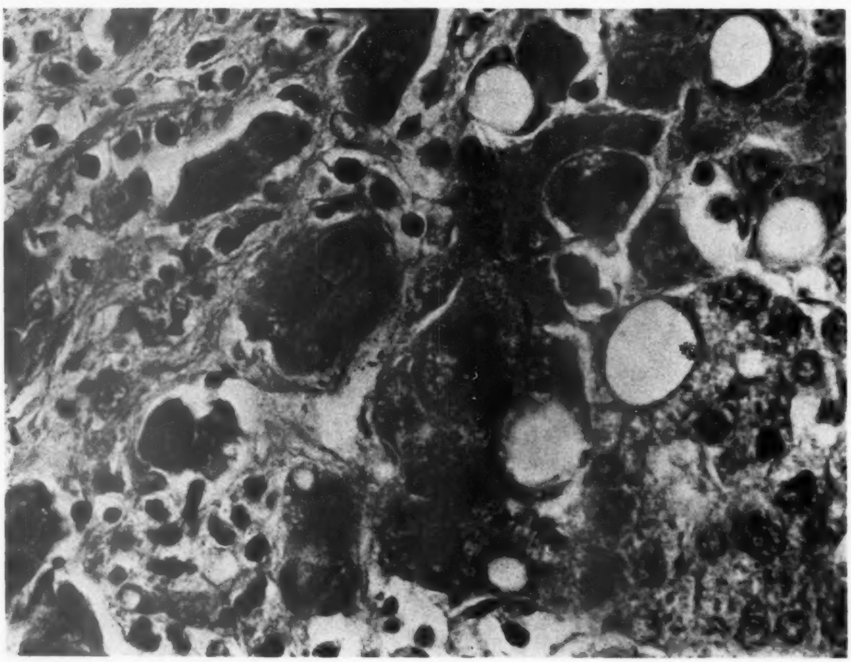
2

FIG. 3. Formalin-fixed liver, stained with chromotrope 2R-aniline blue stain. Note the dark central portion of the intracytoplasmic hyalin. × 600.

FIG. 4. Formalin-fixed section of liver stained with Lillie's myelin stain. Hyalin is prominent. × 700.



3



4

BRONCHIAL ATROPHY AND COLLAPSE IN CHRONIC OBSTRUCTIVE PULMONARY EMPHYSEMA

ROBERT R. WRIGHT, M.D.

From the Department of Pathology and the Cardiovascular Research Institute, University of California Medical School, San Francisco, Calif.

Chronic obstructive pulmonary emphysema is a degenerative lung disease of unknown etiology and obscure pathogenesis. The outstanding clinical symptom is dyspnea, characterized primarily by difficulty in expressing air from the lungs during the expiratory phase of ventilation. This retardation of expiratory air flow is one of the major functional manifestations of the disease.

The cause of this impairment to air flow has long been a subject for controversy. Laennec[1] in "A Treatise on the Diseases of the Chest and on Mediate Auscultation," expressed the opinion that in this disorder a partial obstruction of the smaller bronchi and bronchioles by inspissated mucus, or swollen mucous membranes, produced a check-valve phenomenon. This occurred because normal, strong, active inspiration causes incoming air to by-pass these obstructions, while the weak, passive expiration allows outgoing air to be "trapped" within the alveoli. The role of this difference between the force of the inspiratory and expiratory movement is now considered to be only a minor factor in the mechanism of the obstruction. Other explanations of the expiratory obstruction have been proposed recently, and these are based on two concepts. According to the first, derived primarily from morphologic studies, the underlying cause is considered to be chronic bronchiolitis.[2,3] This is manifested histologically by scarring, narrowing, or obliteration of the bronchioles. The second concept, based essentially upon investigations of pulmonary function, is that the bronchioles collapse during the expiratory phase of ventilation.[4-7] The collapse is considered to be due to pathologic alterations in the elasticity of the lung, upon which the patency of the bronchioles depends. These explanations propose that the obstruction to air flow is confined to the bronchioles. The remainder of the bronchial tree has heretofore been ignored in this disease, except for the clinical observations on the expiratory collapse of the trachea and large bronchi due to the relaxation of the *pars membranacea* in severe emphysema.[8]

This investigation was supported by research grants from the California Tuberculosis and Health Association and the University of California Committee on Research.

Received for publication, October 5, 1959.

The present study represents a morphologic comparison of the bronchi in normal and emphysematous lungs. The investigation demonstrates the participation of the bronchi in the obstructive phenomena in advanced stages of the disorder.

Material and Methods

The lungs from 20 patients with severe, chronic obstructive emphysema as the underlying cause of death and from 20 patients with no pulmonary disease were obtained at necropsy. The ages of the first group ranged from 46 to 71 years and of the second from 43 to 77 years (Table I). All of the specimens examined were from males. The lungs were fixed at necropsy by bronchial infusion of 10 per cent neutral formalin at a pressure of 10 to 15 cm. of water until the normal contours of the lobes were established. After fixation, the bronchial trees of these lungs were dissected from the other pulmonary structures and compared grossly and histologically. Sections were stained with hematoxylin and eosin and the Verhoeff-van Gieson connective tissue stain.

Table I

COMPARISON OF AGES OF PATIENTS WITH NORMAL LUNGS AND THOSE WITH PULMONARY EMPHYSEMA

Age ranges	Normal	Chronic obstructive emphysema
41–45	1	0
46–50	5	3
51–55	3	2
56–60	2	4
61–65	2	4
66–70	2	5
71–75	4	2
76–80	1	0
Total no. of cases	20	20
Mean age	59	61

Observations

There were notable gross and histologic differences between the bronchial trees of the emphysematous and normal lungs. These differences were most readily observed in the medum-sized bronchi (the first, second and third order bronchi, distal to the segmental bronchus). Grossly, the normal medium bronchi were uniformly tapered, tubular structures composed of dense fibrous tissue which contained the usual white cartilaginous plates of various sizes and shapes (Figs. 1 and 2). The lumens remained patent unless external pressure was applied to their walls. Normal bronchial components were microscopically observed (Figs. 5

and 6). Slight age differences were noted: the medium-sized bronchi in the normal lungs of older patients had slightly thinner walls and less cartilage than those of younger individuals. The medium-sized bronchi in emphysema, however, were strikingly different from those in normal lungs (Figs. 3 and 4). They were thin-walled, semitranslucent, collapsed, and gave the appearance of ribbons in contrast to the normal tubular air-conducting bronchi. The cartilaginous plates at this bronchial level were notably reduced in size and number, and wide expanses of "paper-thin," unsupported bronchial wall were commonly observed. These bronchi remained collapsed when dissected free of the surrounding parenchyma. This was the case only in the small bronchi of the normal lung. Ectasia of varying degrees became apparent when the lumens of these collapsed medium and small bronchi were opened. The microscopic examination of the walls of the medium bronchi revealed thin mucous membranes and decreased amounts of fibrous connective tissue, cartilage and smooth muscle (Figs. 7 and 8). The fibrous tissue was condensed and composed of attenuated bundles of collagen fibers closely interwoven about the cartilaginous plates. The cartilages were small, and in some areas there was apparent replacement by fibrous tissue. The remaining cartilaginous matrix and cells were normal, except for the commonplace calcification in the centers of the plates seen in the older patients in both the normal and the emphysema groups. The bronchial smooth muscle was sparse and the spiral bundles widely separated. The mucous membranes possessed a thin lamina propria, and the longitudinal folds were frequently absent and represented by mere undulations of the respiratory epithelial layer. Although the mucous glands were occasionally dilated and filled with mucus, their numbers appeared to be reduced.

There were grossly detectable differences between the large bronchi (main stem, lobar and segmental bronchi) of the emphysematous lungs and the normal lungs, although these were less obvious than those in medium bronchi. The large bronchi in emphysematous lungs frequently possessed elliptical rather than round lumens, and the membranous portions of the main stem bronchi were often broad, thin and flaccid (Fig. 9). These were more easily collapsed by external manual pressure than were the normal bronchi. Microscopically, the alterations were similar to those noted in medium bronchi, but were not so marked.

The small bronchi (2 mm. or less in diameter) were thin-walled and remained collapsed in both groups. Ectatic bronchi in the emphysematous lungs were usually extremely thin. Although small cartilages were seen microscopically in the normal small bronchi, they were diminished in size and number, and, occasionally, were completely absent at this

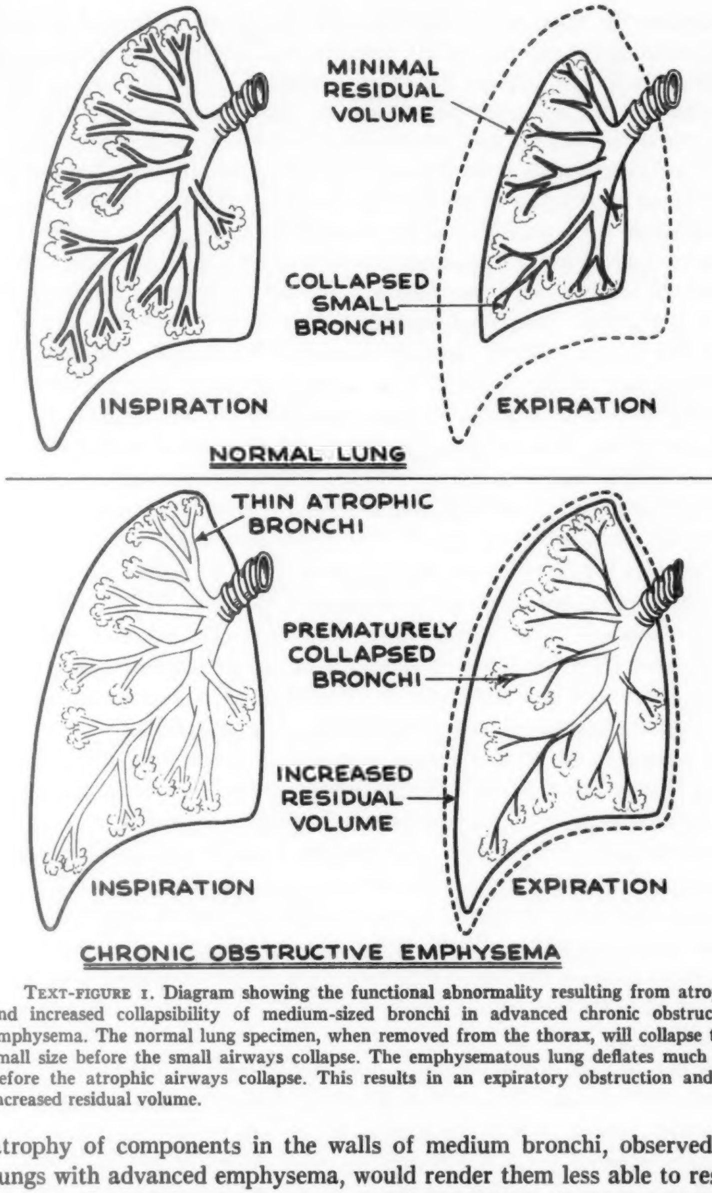
bronchial level in the emphysematous specimens. Other microscopic features were again similar to those observed in medium bronchi, but were less marked.

These alterations in the bronchi of emphysematous lungs are best designated as atrophy. There was minimal evidence of active, chronic bronchitis other than the presence of occasional foci of squamous metaplasia and an increased number of goblet cells in the respiratory epithelium. Acute purulent bronchitis encountered in some cases of emphysema was considered to be a terminal complicating event and not of pathogenic significance. In most cases the walls of the bronchial arteries were thick, muscular and fibrous, and the lumens were narrowed; however, since this was seen in some of the normal lungs of older patients, its significance could not be evaluated.

Discussion

These studies indicate that atrophy of the walls of bronchi in emphysematous lungs is a significant factor in the expiratory obstruction in the advanced disease. The act of breathing produces slight air pressure changes between the outside and the inside of the intrapulmonary bronchi.[9] During inspiration the expanding thorax causes the intra-alveolar air pressure to fall below the level of the air pressure within the bronchi, and air flows from the bronchi into the alveoli. During expiration the process is reversed; intra-alveolar pressure rises above intrabronchial pressure, and air passes into the bronchi and out the upper respiratory tract. The intrapulmonary position of the segmental, medium and small bronchi exposes the walls of these bronchi to this continuously alternating transmural air pressure gradient. Thus the bronchi tend to dilate during inspiration and become narrow during expiration. These airway changes are opposed by the normal bronchial structures and also by the elastic forces of the normal lung. The large bronchi depend primarily on cartilaginous rings and plates, and dense fibrous tissue content for support. The medium bronchi depend partly on the rigidity of their walls and partly on the radial traction forces of the surrounding expanded lung tissue. The small bronchi and bronchioles are almost entirely dependent on the latter.

Quiet respirations probably produce insignificant changes in airway diameters, but rapid ventilation, particularly forced expiration such as a cough, markedly increases the pressure difference between the inside and the outside of the intrapulmonary bronchi. The airways in the normal lung collapse partially during a strenuous cough.[10,11] Thus, the integrity of the bronchial wall is evidently important in maintaining the patency of the large and medium bronchi during expiration. The marked



TEXT-FIGURE 1. Diagram showing the functional abnormality resulting from atrophy and increased collapsibility of medium-sized bronchi in advanced chronic obstructive emphysema. The normal lung specimen, when removed from the thorax, will collapse to a small size before the small airways collapse. The emphysematous lung deflates much less before the atrophic airways collapse. This results in an expiratory obstruction and an increased residual volume.

atrophy of components in the walls of medium bronchi, observed in lungs with advanced emphysema, would render them less able to resist collapse during the expiratory phase of ventilation, thereby producing the expiratory air flow obstruction, so characteristic of the disorder (Text-fig. 1). The elastic traction forces of the lung parenchyma, which

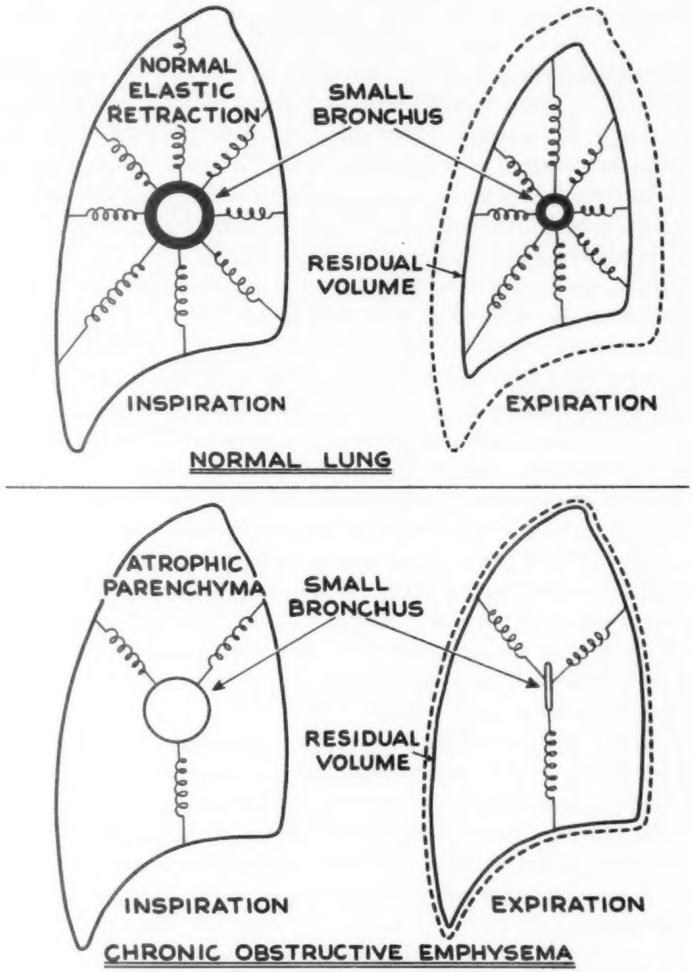
maintain the patency of small bronchi and bronchioles and assist in maintaining the patency of the medium bronchi, were not evaluated in this study. In emphysema the elastic properties of the pulmonary tissue are altered in a manner which allows the small airways to collapse during the expiratory air flow and thereby contributes to the obstruction.[12]

Another symptom of the disease is wheezing, and this may be partially explained by the flow of air through the thin, collapsed bronchi during exhalation. Accumulations of inspissated secretions within the bronchi also contribute to the wheezing. Bronchial spasm does not seem to be a likely factor in view of the atrophic musculature in the advanced stage.

A prominent functional manifestation of the disorder, increased residual volume, may be partially caused by the atrophy and increased collapsibility of the bronchi. The normal, air-filled lung specimen, if allowed to deflate, will not collapse completely but will retain a certain amount of air. This retention is due to the diminished radial traction forces of the lung parenchyma in the deflated state, and also to the collapse of the small airways which depend upon radial traction to hold their lumens open (Text-fig. 2). The small lung volume which allows the airways to collapse is not reached in the living normal person because the chest wall cannot contract to this degree. However, if the bronchi are more collapsible because of atrophy of their mural components and loss of support from the lessened elasticity of the emphysematous parenchyma, the medium as well as the small bronchi will collapse prematurely during the expiratory phase (Text-fig. 2). A large residual volume will then remain in the lung. Also, the necessity for the patient with emphysema to ventilate at abnormally large lung volumes would be explained by the effectiveness of pulmonary overdistention in providing increased radial traction on the walls of the atrophic bronchi and thereby opposing the premature collapse of the airways during expiration.

High ventilatory rates and forced expirations, such as cough, are opposed by the atrophic, collapsible bronchi in emphysema because these functions increase the transmural bronchial air pressure gradients. The weakened bronchial walls therefore collapse and obstruct during the period of expiratory air flow.

In the present investigation it was noted that in emphysema the length and severity of such predisposing diseases and conditions as bronchial asthma, chronic bronchitis, cough and exposure to bronchial irritants differed in duration and severity. Morphologic distinctions could not be made in the end stages of the disorder. Atrophy of the bronchial walls may be a common result of a variety of bronchial insults. Not apparent in this study is the role of atrophy of the bronchial tree in the pathogenesis of the disease. Bronchial atrophy may be only part



TEXT-FIGURE 2. Diagram showing the contributory role of the radial traction forces of the lung parenchyma in maintaining the patency of the medium and smaller airways (including the bronchioles) during the expiratory phase of ventilation. The atrophic parenchyma of the emphysematous lung fails to provide adequate circumferential traction on the walls of these airways. This is an additional factor in allowing premature collapse of the bronchi, which results in obstruction to expiratory air flow.

of a generalized atrophy of the lung; consequently the expiratory obstruction is merely one manifestation of the condition. Another possibility is that certain bronchial diseases predispose to bronchial atrophy and collapse; the resulting obstruction to expiratory air flow in turn leads to emphysematous disintegration of the lung parenchyma by unknown means.

Summary

Previous explanations of the expiratory air flow obstruction in chronic obstructive pulmonary emphysema imply that only the smaller airways are affected. Morphologic observations of other portions of the intrapulmonary bronchial tree have not heretofore been made. Specimens from the bronchial trees of 20 normal lungs and 20 with advanced emphysema, fixed in formalin, were dissected free of surrounding pulmonary parenchyma and compared morphologically.

The differences between the two groups of bronchi were most notable in the medium-sized bronchi (first, second and third order bronchi, distal to the segmental bronchi). These medium bronchi in emphysematous lungs were collapsed, thin-walled, and showed marked reduction in cartilage and connective tissue content. The features were indicative of severe bronchial atrophy. Only minimal evidence of active chronic bronchial inflammation could be seen in the advanced cases.

In advanced pulmonary emphysema, the characteristic expiratory obstruction may be due to collapse of the atrophic airways during expiration. The emphysematous degeneration of the parenchyma and the lessened radial traction on the airways would allow collapse of small bronchi and bronchioles, thus contributing to the obstruction. The collapse of the bronchi may also contribute to such manifestations as increased residual volume, wheezing respiration, and the necessity for ventilation at abnormally large lung volumes.

Bronchial atrophy may be merely a component of the generalized atrophy of the lung in emphysema. On the other hand, it may be the end result of bronchial insults such as inflammation, coughing, or contact with irritants. Either could lead to bronchial collapse and expiratory air flow obstruction. Chronic expiratory obstruction, whatever its cause, may in turn induce emphysematous degeneration of the pulmonary parenchyma.

References

1. Laennec, R. T. H. A Treatise on the Diseases of the Chest and on Mediate Auscultation. Forbes, J. (transl.). S. S. & W. Wood, New York, 1838, ed. 3, pp. 167–168.
2. Spain, D. M., and Kaufman, G. The basic lesion in chronic pulmonary emphysema. *Am. Rev. Tuberc.*, 1953, **68**, 24–30.
3. McLean, K. N. The pathogenesis of pulmonary emphysema. *Am. J. Med.*, 1958, **25**, 62–74.
4. Dayman, H. Mechanics of airflow in health and in emphysema. *J. Clin. Invest.*, 1951, **30**, 1175–1190.
5. Comroe, J. H., Jr.; Forster, R. E.; Dubois, A. B.; Briscoe, W. A., and Carlsen, E. The Lung: Clinical Physiology and Pulmonary Function Tests. Yearbook Publishers, Chicago, 1955, p. 123.

6. Mead, J.; Lindgren, I., and Gaensler, E. A. The mechanical properties of the lungs in emphysema. *J. Clin. Invest.*, 1955, **34**, 1005–1016.

7. Fry, D. L.; Ebert, R. V.; Stead, W. W., and Brown, C. C. The mechanics of pulmonary ventilation in normal subjects and in patients with emphysema. *Am. J. Med.*, 1954, **16**, 80–97.

8. Herzog, H. Exspiratorische Stenose der Trachea und der grossen Bronchien durch die erschlaffte Pars membranacea. Operative Korrektur durch Spanplastik. *Thoraxchirurgie*, 1958, **5**, 281–319.

9. Dayman, H. G. Obstructive Conditions of the Respiratory Tract (Fundamental). In: Clinical Cardiopulmonary Physiology. Gordon, B. L. (ed.). Grune & Stratton, Inc., New York, 1957, pp. 246–252.

10. Dekker, E.; Defares, J. G., and Heemstra, H. Direct measurement of intrabronchial pressure. Its application to the location of the check-valve mechanism. *J. Appl. Physiol.*, 1958, **13**, 35–41.

11. DiRenzo, S. Bronchial dynamism. *Radiology*, 1949, **53**, 168–186.

12. Stead, W. W.; Fry, D. L., and Ebert, R. V. The elastic properties of the lung in normal men and in patients with chronic pulmonary emphysema. *J. Lab. & Clin. Med.*, 1952, **40**, 674–681.

[*Illustrations follow*]

Legends for Figures

Photomicrographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Dissected bronchial tree of normal left upper lobe, 36-year-old male. Many of the smaller bronchi have been sacrificed necessarily in the dissection. Note the tubular, tapering bronchi with grossly observable cartilages extending peripherally to the level of the arrow.

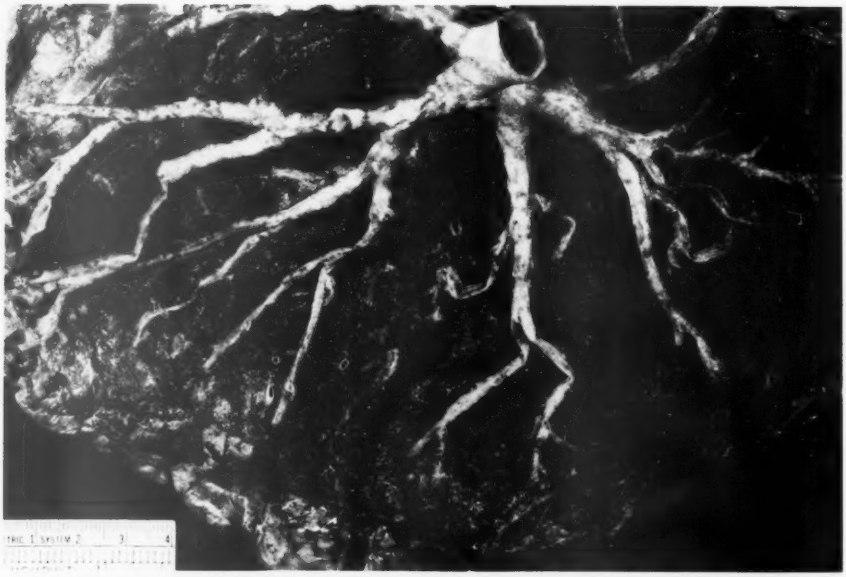
Fig. 2. Bronchial tree of normal left upper lobe, 77-year-old male. Slight atrophy of the walls of the medium-sized bronchi, common in elderly persons, can be noted.

1

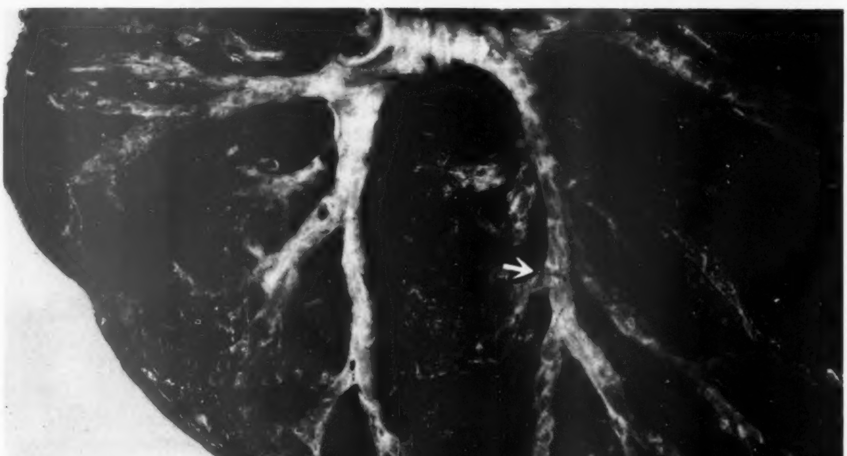
2

FIG. 3. Bronchial tree of emphysematous left upper lobe, 49-year-old male. The medium-sized bronchi are thin-walled and partially collapsed; the amount of supporting cartilage is reduced.

FIG. 4. Bronchial tree of lung with severe emphysema, 62-year-old male. The medium-sized bronchi are markedly thin-walled, collapsed and ectatic. Cartilage cannot be seen grossly, peripheral to the level of the arrow.



3



4

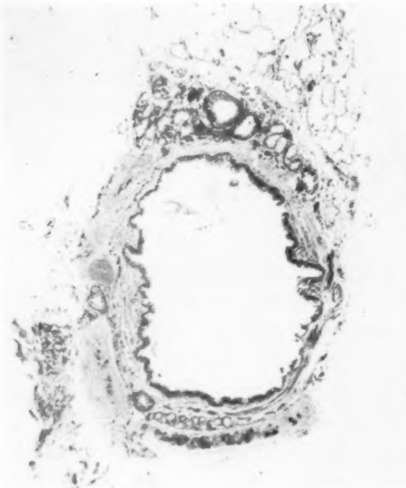
FIG. 5. A normal medium-sized bronchus (second order, distal to the segmental bronchus). Note the thick, well supported wall. × 16.

FIG. 6. The same bronchus shown in Figure 5. Note the normal components of the bronchial wall including the mucous membrane. × 48.

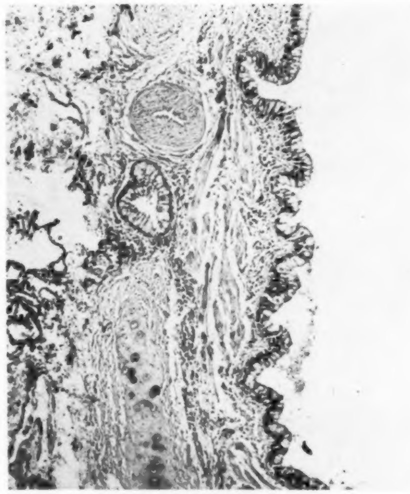
FIG. 7. A medium-sized bronchus (second order, distal to the segmental bronchus) in pulmonary emphysema. Note the thin, atrophic wall with diminished content of cartilage, muscle and connective tissue. × 16.

FIG. 8. The same bronchus shown in Figure 7. Note the severe atrophy of the muscle, cartilage, connective tissue and mucous membrane. × 48.

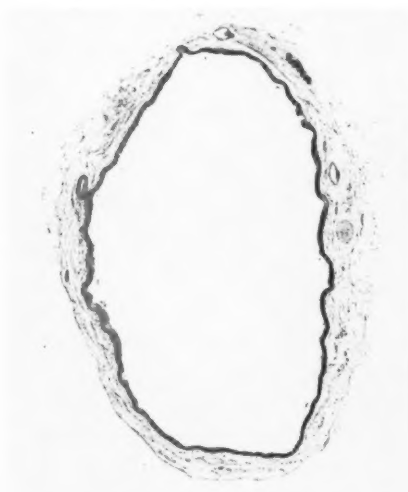
FIG. 9. A cross section of a main stem bronchus in a normal (left) and an emphysematous lung (right). Note the wide "c" rings and flaccid membranaceous portion of the bronchus in the emphysematous lung. × 2.



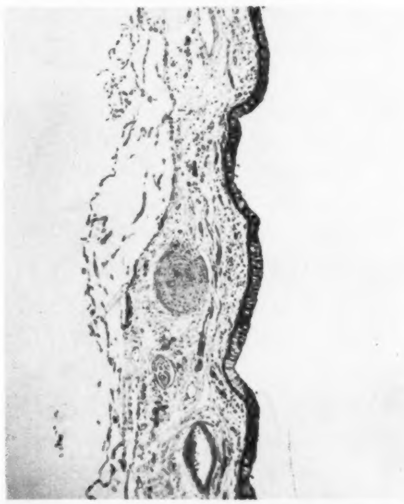
5



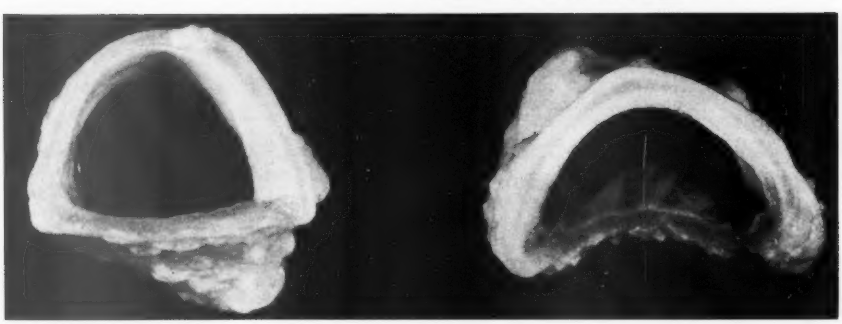
6



7



8



9

THE EFFECT OF A TOXIC CELLULAR COMPONENT OF GROUP A STREPTOCOCCI ON CONNECTIVE TISSUE

WILLIAM J. CROMARTIE, M.D.; JOHN H. SCHWAB, PH.D., AND JOHN G. CRADDOCK, M.D.

From the Departments of Bacteriology and Medicine, University of North Carolina School of Medicine, Chapel Hill, N.C.

The physical, chemical, and biologic properties of cellular components derived from sonic-disrupted group A streptococci have been recorded in previous reports.[1-6] One of the biologic properties of the sonic extract is its ability, following a single intradermal injection into rabbits, to produce a chronic remittent and intermittent reaction in the dermal connective tissue, characterized by the formation of multinodular lesions.[3,4] The injection of extracts of streptococci other than group A failed to produce a comparable reaction.[5] The cellular component responsible for this toxic activity has been isolated and shown to be a macromolecular complex of group-specific C polysaccharide and a peptide.[6]

The present report presents further observations on the course of this reaction extending over a 60-day period; a more detailed description of the histologic alterations associated with the various stages of the process than was previously reported[3,4]; further observation on lesions induced by injection of similar extracts of a variety of streptococci other than those of group A; and an evaluation of the role of hypersensitivity in the tissue injury observed.

MATERIAL AND METHODS

Extraction of Cells

The cultivation, harvesting, and extraction of the group A streptococcal cells have been described in previous communications.[1,3] In the experiments reported here, the washed cells from 1 liter of an 18-hour broth culture were suspended in 15 ml. of a phosphate buffer, pH 7.0, and subjected to sonic vibration in a Raytheon 9 kc. sonic oscillator for a period of 1 hour. The material was then centrifuged in a Spinco preparative centrifuge at 36,000 × G for 30 minutes, the supernatant fluid filtered through a Selas 02 filter and frozen at −20°C. in sealed ampules. The sterility of each preparation was confirmed by inoculating thioglycolate broth and blood agar plates with 0.5 ml. aliquots. Extracts

This investigation was supported by a research grant (E-949) from the National Institute of Allergy and Infectious Diseases, United States Public Health Service.

Received for publication, October 26, 1959.

of the various strains of organisms were standardized on the basis of total nitrogen content.

Organisms

A strain of a type 1 group A streptococcus (*Streptococcus pyogenes*) was the organism used. Extracts, prepared in a similar manner, of the following organisms were used for comparative purposes: a group B streptococcus (*Streptococcus agalactiae*); a group D streptococcus (*Streptococcus durans*); a viridans streptococcus (*Streptococcus mitis*).

Animals

New Zealand white rabbits, weighing 2.0 to 3.0 kg., which were obtained from a single commercial breeder, were used throughout these experiments.

Preparation of Tissues

Sections for histologic examination were fixed in 10 per cent formalin solution. Paraffin sections were stained with the following methods: hematoxylin and eosin; phosphotungstic acid hematoxylin; luxol fast blue-periodic acid-Schiff-hematoxylin stain[7]; a Masson type of trichrome stain described by Gomori[8]; the pentachrome I method of Movat[9]; and Gomori's silver impregnation technique.[10]

RESULTS

Reaction Produced by a Single Intradermal Injection of an Extract of Group A Streptococci

For the purpose of studying in more detail and for a longer period of time the course of this reaction, 86 rabbits received intradermal injections, at a single site in the flank, of 0.2 ml. of the extract of the type 1 group A streptococcus. Fifty of these animals were sacrificed at various intervals after injection. The remaining 36 animals were observed for a total of 60 days.

The general gross features of the lesions were as previously described.[3,4] Three hours after injection, a moderate thickening and erythema was noted in the skin of all animals at and around the site of injection. These initial lesions varied in size from 20 by 20 mm. to 120 by 60 mm. and were elevated 1 to 2 mm. above the skin surface. The edema and erythema reached a maximum intensity in 12 to 24 hours after injection and persisted for varying periods of time. In 40 of the 62 animals observed for 10 days, the initial reaction subsided completely 48 hours after injection, and the skin at and around the injection site in this group appeared normal until the nodular lesions devel-

oped. In 22 animals the initial thickening and erythema at the site of injection persisted for as long as 14 days, and the nodular lesions developed before the initial reaction had completely subsided. The time of appearance of the first group of nodules in each animal was observed to vary from 2 to 14 days. In a single animal a nodular lesion was not observed until 30 days after injection.

As previously described, the lesions consisted of rounded nodules and elongated ridge-like elevations.[3,4] The rounded nodules measured 8 by 8 to 30 by 30 mm. and projected 2 to 15 mm. above the skin surface. The ridge-like elevations measured 15 to 20 mm. in width and 30 to 50 mm. in length and projected as much as 15 mm. above the skin surface. The number of distinctly separate nodules observed in a single rabbit varied from 4 to 46. As the process evolved, the isolated lesions tended to coalesce, and elevated nodular plaque-like areas, measuring from 40 by 30 mm. to 60 by 120 mm. and projecting 10 to 15 mm. above the skin surface, were formed. The nodular lesions reached their maximum size 24 to 48 hours after they were first observed. At this time they were of a soft consistency with marked erythema of the overlying skin surface. Necrosis of the skin or hemorrhage into the tissues was not a feature of the lesions.

The course of the reaction was observed for 60 days in 36 rabbits and was noted to vary rather markedly in individual animals; however, 3 general patterns of evolution were observed which may be outlined as follows: (1) The soft, edematous, nodular lesions with associated erythema of the overlying skin subsided in 48 to 72 hours, leaving a firm, slightly elevated, irregularly shaped plaque-like lesion which gradually completely disappeared over a period of 10 to 12 days, and no additional changes were observed. The lesions in 3 of the 36 animals were observed to follow this pattern as they evolved. (2) The course of the reaction in 22 of the animals was similar to the first pattern described except that as the plaque-like lesions were subsiding, new nodules developed in the areas in which the process was subsiding. These new nodules were of the same gross appearance as the initial nodules and subsided in a similar manner in 48 to 72 hours, leaving the firm pale plaque-like elevations that gradually disappeared in 7 to 21 days. From 1 to 5 such remissions and exacerbations were noted in each animal in this group. Exacerbations were noted between 10 and 45 days after injection. In 10 of these animals, pale, plaque-like lesions were present at the time the experiment was terminated. (3) In 11 rabbits the pattern of reaction differed from those described above in that the initial nodular lesions subsided completely after 7 to 21 days, and new nodules developed in the previously involved areas. From 1 to 4 such recurrences were noted in each animal in this group.

These recurrent lesions developed between the 16th and 53rd day after injection. Figure 1 illustrates a recurrent lesion that developed on the 17th day after the animal was injected. The recurrent nodules were in general smaller than the original nodules, varying in size from 8 by 8 to 25 by 25 mm. and were elevated 8 to 10 mm. above the skin surface. From 2 to 8 such nodules were noted with recurrence of the process. In 4 of these animals subsiding lesions were present at the time the experiment was terminated.

Microscopic Observations. The initial diffuse thickening and erythema of the skin noted 3 hours after injection of the extract was associated with a phlegmonous type of acute inflammatory reaction. Sections extending through the point of injection showed a diffuse change characterized by separation of the collagen bundles and infiltration with heterophils.[11] In the area of acute inflammation the collagen bundles showed a wide range of alterations, varying from minimal swelling to what appeared to be complete dissolution. In addition to the rather diffuse changes, focal areas were infiltrated by necrotic appearing leukocytes. The collagen bundles in these focal lesions were replaced by a mixture of fibrillar, granular and homogeneous amorphous material (Fig. 2).

The histologic alterations observed in sections of the nodular lesions fixed at different periods after injection of the extract seemed to reflect the age of individual nodules within the total area of involvement. The microscopic alterations were similar in nodules that had been visible in the gross for approximately the same period of time. In other words, nodules that had been present for 24 hours when fixed showed similar histologic alterations whether the nodule had appeared in the gross 72 hours after injection or as late as 52 days after the injection was made. However, the nodular lesions which developed at 3 to 8 weeks were not as large as similar lesions which developed during the first 2 weeks after injection. There seemed to be a gradual reduction in the intensity of the recurrent injury as the process evolved. This was reflected in the microscopic changes observed. In general the foci of necrosis were smaller in the lesions which developed late in the course of the process.

Nodular lesions procured 24 to 48 hours after they had appeared showed what seemed to represent progression of the reaction observed 3 hours after injection of the extract. The diffuse phlegmonous reaction was associated with more marked alteration of the collagen bundles, and the foci where collagen was replaced by fibrillar, granular and homogeneous amorphous material were increased in size. In some areas fibrillar eosinophilic-staining material formed an interlacing network which replaced the collagen bundles.

The fibrillar, granular, and homogeneous amorphous substance exhibited the staining reactions usually ascribed to fibrinoid.[12] It was stained red by the trichrome method; rose to pink by the luxol fast blue-periodic acid-Schiff-hematoxylin stain; red by the pentachrome I method; and dark purple to blue by the phosphotungstic acid-hematoxylin method. After silver impregnation the fibrillar material was blackened, as noted by Glynn and Loewi in their studies of fibrinoid.[13] In other regions the connective tissue of the dermis was replaced by dense masses of necrotic heterophils. What appeared to be scattered remnants of collagen bundles were observed among the necrotic cells. This lesion, which resembled an abscess in some respects, seemed to represent the reaction to the most severe degree of injury whereas in other areas in the same section the reaction consisted of minimal swelling of the collagen bundles. A striking feature of the process was the wide spectrum of histologic changes seen in a single section. Figures 3 to 8 illustrate the varied histologic features in nodular lesions obtained 24 to 48 hours after they appeared in the gross.

Sections of nodular lesions developing as a relapse of a lesion which had appeared to subside completely, or as an exacerbation of a subsidiary lesion, showed chronic infiltrative and proliferative reactions in addition to the areas of necrosis and associated acute inflammatory reaction. Illustrations of this combination of acute and chronic reactions are shown in Figures 3 and 8.

The chronic reaction which was seen in all lesions procured 6 to 7 days after they appeared was characterized by a variety of histologic changes seen in different portions of the same section. These included small scattered collections of lymphocytes; diffuse and focal infiltration by macrophages; focal accumulations of macrophages and giant cells; and reactions characterized by a combination of fibroplasia, capillary formation, and accumulations of lymphocytes, macrophages and giant cells. The multinucleated cells were quite variable in appearance. In some the nuclei were arrayed around the cytoplasmic periphery; in others these were more central in position. In all, the nuclei seemed to contain rather large masses of chromatin which in some cells appeared as a dark round mass in the center of the nucleus and in others as a long bar of dark substance. The cytoplasm of a few of these cells was sharply defined whereas that of a majority was very irregular in outline. The cytoplasm of the giant cells appeared basophilic. Many of the large macrophages appeared to contain a mass of homogeneous eosinophilic-staining cytoplasmic substance. Many of the giant cells and macrophages appeared in association with fragments of collagen bundles. Figures 9 and 10 illustrate the various patterns of chronic inflammation observed.

As indicated above, in certain sections fixed shortly after an exacerbation or a relapse had occurred, the chronic proliferative reaction was associated with foci of necrosis and acute inflammatory reaction; in other sections fixed at a time when the lesion appeared to be stable or to be subsiding grossly, only the chronic type of granulomatous reaction was noted.

The acute and chronic inflammatory lesions extended throughout the dermis. They seemed to be most marked in the deep portion adjacent to the *panniculus carnosus*. This layer of muscle was occasionally affected by the acute inflammatory reaction. A feature of the process was the rather minimal involvement of the blood vessels. Evidence of thrombus formation was not a characteristic at any stage and only in the areas of severe diffuse acute inflammation was acute inflammation of the vessel walls noted. The changes in the dermal connective tissue did not appear to result from primary injury of blood vessels. In a few areas a moderate degree of endothelial hyperplasia of small arteries was noted in the chronic lesions.

The inflammatory process tended to subside rather slowly. In none of the animals investigated by histologic methods had the reaction completely subsided when the sections were fixed. This included the animals examined 53 days after injection. Three animals were sacrificed at a time when the skin at and around the injection site for 30 to 50 mm. had appeared normal in the gross, except for slight induration, for 1 to 3 weeks. Sections from these sites revealed foci of macrophages and lymphocytes scattered throughout the dermis.

Effects of Viable Group A Streptococci and Extracts of Streptococci Other Than Group A

Comparable extracts of the following organisms were prepared as described above: *Streptococcus agalactiae* (group B); *Streptococcus durans* (group D); and *Streptococcus mitis*. Each of 18 rabbits received intradermal injections in different sites in the flank region (0.2 ml. of each of the extracts, 0.2 ml. of group A extract, and at a fifth site approximately 2×10^9 viable cells of the group A strain). Two rabbits were sacrificed for histologic examination at the following periods after injection: 3 hours, 1, 3, 7, 14, 21, and 30 days. Six rabbits were observed for 30 days at which time the experiment was terminated. The 12 animals observed for 7 days or longer all exhibited the characteristic remittent and intermittent multinodular lesions in the areas injected with extracts of the group A organism. The gross and microscopic features of these lesions were the same as those observed in the 86 animals described above.

In the areas injected with extracts of ***Streptococcus durans*** (group D) and ***Streptococcus mitis,*** a single area of edema and erythema developed around the site of injection. These lesions reached a maximum size between 24 and 48 hours and gradually subsided during a 10-day period. No nodules developed and no recurrences or exacerbations of the lesions were observed. The maximum area of skin involvement by the edema and erythema varied from 20 by 20 mm. to 50 by 60 mm. In the region where the extract of ***Streptococcus agalactiae*** (group B) was injected, lesions similar to those resulting from the extracts of group D and the ***Streptococcus mitis*** organisms were observed, with the exception that in 3 animals, as the edema and erythema subsided, 3 to 6 small nodules, 10 by 10 mm. in size, were observed around the injection site. In 2 of these 3 animals the nodular lesions increased in size between 7 and 14 days after injection. In 1 additional animal, 14 days after injection, a lesion consisting of 15 small, 8 by 8 mm. nodules developed in an area where the initial edema and erythema had completely subsided. These were the only examples of the appearance of gross lesions resembling the remittent and intermittent multinodular reactions observed at sites injected with the extracts of the group A organisms.

The viable group A cells produced extensive areas of edema and erythema followed by ulcer formation. The area of skin involvement varied from 60 by 78 mm. to 110 by 40 mm. An eschar developed in the center of the lesions by the fourth day. When this was removed, an ulcerated surface measuring 20 by 35 mm. in diameter was exposed, from which a moderate amount of purulent material exuded for 3 to 4 days. Healing of these ulcers grossly appeared to be completed by the 21st day in all animals, leaving a moderately indurated area of scar formation that measured from 10 by 15 to 20 by 30 mm. in the different animals.

Microscopic examination of these reactions may be summarized as follows: The microscopic alterations in response to extracts of group A cells were the same as those encountered in the animals described above. The lesions which developed at the sites where the 3 other extracts were injected were associated with a phlegmonous type of acute inflammation similar to that observed 3 hours after the extracts of group A organisms were injected. However, the alteration of the collagen bundles was never marked, and the combination of necrosis and chronic inflammatory reaction noted at sites of group A streptococcal extract injection was not encountered (Fig. 11). The healing process appeared to be continuous and was complete 21 days after injection. It should be noted that the experimental design did not include histologic examination of the nodular lesions which appeared at 4 of the sites injected with the group B extract.

Sections from the sites where the viable cells were introduced revealed extensive necrosis and abscess formation followed by microscopic evidence of ulceration. Gradual healing was associated with rather extensive scar formation. No evidence of recurrent acute necrosis was noted in the healing lesions. Histologic studies, 21 and 30 days after injection, of the sites where viable cells were introduced revealed evidence of minimal chronic inflammation. This was indicated by a few focal collections of macrophages and an occasional giant cell in association with a rather marked degree of fibroplasia.

Effects of Multiple Injections of Extracts of Group A Streptococci

To investigate the role of hypersensitivity in the reaction to the extracts of the group A streptococci, each of 28 rabbits received intradermal injections with 0.2 ml. of the extract. Groups of 3 of these animals were given intradermal injections in the opposite flank region at 1, 2, 3, 4, 7, 8 and 12-day intervals after the first injection. The course of the reaction was observed, in the gross, for a period of 30 days after the second injection. Another group of 63 animals also received intradermal injections with 0.2 ml. of the extract. Twenty-five of these were given a second injection 60 days after the first, and 13 received a third injection 50 days after the second. At the time the second and third injections were made, an equal number of normal rabbits received the same extract. Eight animals were sacrificed for microscopic study at intervals between 3 hours and 30 days after the second injection. At the same time 8 of the control animals that had received only one injection were also sacrificed. One experimental and one control animal were killed after 1, 2, and 7-day intervals, following the third injection.

Both gross and microscopic examination showed that the development and evolution of the process was not modified by multiple injection. The time of appearance and the course of the lesions were similar whether subsequent injections were given before the appearance of the nodular lesions resulting from the first injection, immediately after the appearance of the lesions, or after they had subsided. No modification of the histologic reaction was noted in the lesions resulting from second or third injections. The absence of thrombosis and necrosis of blood vessels was a feature of the lesions resulting from second and third injections, as it was in the lesions resulting from first injections (Fig. 12).

Discussion

The immediate injury of dermal connective tissue, the intermittent and remittent character of the course, and the rather long duration of

the process are striking features of the reaction to a single injection of the extract of group A streptococcal cells. The observations recorded in other reports regarding the physical and chemical nature of the toxic material in this extract[6,14] suggest a mechanism of tissue injury and factors which may relate to the long duration of the process. The uniform nature of the overall pattern of the reaction observed in all the animals injected with the group A streptococcal extract, combined with the results of the reinjection experiments described above and the histologic features of the lesions, tends to exclude a hypersensitivity reaction as the cause of the damage observed. If previous natural contact with similar antigenic substance were responsible for the reaction, one would have to postulate that all the animals injected in this and in previous experiments had developed at least a somewhat similar hypersensitive state due to natural contact with identical or cross-reacting antigenic material. Such an occurrence would seem highly unlikely. The fact that repeated injections did not modify the course of the reaction also makes it appear that a hypersensitivity reaction is probably not responsible for the tissue damage observed. The nature of the histologic lesions, especially the absence of vascular necrosis and thrombosis despite rather extensive necrosis of the dermal connective tissue, would also tend to exclude hypersensitivity as a mechanism.

Physicochemical studies have demonstrated that the toxic activity of the group A streptococcal extract is associated with a rather limited range of particle size of the polysaccharide-peptide complex.[14] This suggests that the physical properties of this material may be of importance. It is known that many substances in a colloidal state will react readily with large polymers resulting in alterations in the physical properties of the colloidal system. Thus, reaction of the streptococcal macromolecule with the biocolloids of connective tissue may result in an alteration of the gel-sol state in essential components of connective tissue with consequent loss of normal structure and function of the tissue. It is suggested that a reaction of this nature may account for the immediate injury observed as early as 3 hours after injection of the group A streptococcal extract.

The course of the reaction extended over a period of at least 60 days following a single injection. The evolution of the process was characterized by exacerbation of subsiding lesions and development of new nodules in areas where, in the gross, the reaction appeared to have completely subsided. This rather long duration of the reaction and the development of acute lesions as late as 53 days after a single injection suggest that the material remains in the tissue and retains its toxic properties for a relatively long period of time. The mechanism that permits

this substance to produce repeated acute injury of the tissue after a single injection is not made evident by these studies. It has been noted that there is a relation between the amount of the toxic material injected and the time of appearance of grossly observable nodular lesions.[6] It was observed that the injection of the higher dilutions of the crude extract was associated with a longer time lapse between the injection and appearance of the nodular lesions than noted when the more concentrated crude extract was injected. It is possible that the exacerbations and relapses encountered represent the late development of lesions at areas where relatively low concentrations of the toxic material accumulated at the time of injection. Another possible explanation of the relapsing nature would be that the toxic material forms an unstable complex with some tissue component, the complex being nontoxic. Subsequent release of the streptococcal component from the complex would account for the development of the acute focal lesions which interrupt healing at various intervals up to at least 53 days after injection. Understanding of the exact mechanisms responsible for this remarkable feature will require further investigation.

The fact that the C polysaccharide which is an essential part of the toxic complex[6] is limited to group A streptococci would explain the failure to obtain material with the same toxic properties from streptococci other than group A. The observations that the extract of the group B organism produced relatively small multinodular reactions in 4 of 10 animals would warrant further study of this group of streptococci to determine the nature of the substance responsible for the lesions.

The ability of certain polysaccharides, such as those in the pneumococcal capsule to remain in the tissues of intact animals for long periods of time is well known.[15] The inability of the rabbit to digest or excrete the toxic macromolecular polysaccharide complex promptly might explain the duration of the process for at least 60 days following a single injection.

The reactions of animals to second and third injections also show that the development of immunity does not play a role in the gradual subsidence of the reaction. Second and third injections at the time the lesions resulting from previous injections were clearly subsiding or appeared in the gross to have completely subsided, produced reactions comparable to those resulting from the first or second injection. Recent studies have shown that group-specific C antibodies will neutralize the toxic effect of this extract.[16] It was also demonstrated[16] that animals rendered hyperimmune by pepsin-treated whole group A cell vaccine, to yield a high anti-C titer, exhibited a degree of immunity against the toxic effects of the extracts. The immunized animals developed small

lesions which healed promptly as compared to the chronic reactions lasting for at least 30 days, observed in the control animals. The results of other studies suggest that the group-specific C-polysaccharide of group A streptococci is a poor antigen in that antibodies against this antigen appear in the serum of a relatively small per cent of patients recovering from an infection with group A streptococci and are rarely found in the serum of patients with acute rheumatic fever.[17,18] In seeking an explanation for the long duration of the toxic effect of this substance after a single injection, the fact that the C-polysaccharide is a poor antigen should be considered along with the fact that the rabbit may be unable to digest or excrete the toxic macromolecular complex promptly. Failure of the antigen in this dose and form to stimulate a significant titer of antibodies probably explains the fact that repeated injections do not seem to modify the course of the reaction.

The C-polysaccharide of group A streptococci has been investigated by Schmidt[19] and Zittle and Harris.[20] Its molecular weight, as determined by both groups, was estimated to be about 8,000. No pathologic lesions were noted when this substance was injected into mice intravenously,[19] and it appeared to be excreted rapidly. Recent studies have related the toxic properties of the specific C-polysaccharide of group A streptococci to the size of the macromolecular complexes that may be prepared.[14] Preparations of C-polysaccharide were procured in a variety of forms, ranging from the simple haptene obtained by formamide extraction to a suspension of purified cell walls, and including fractions of intermediate complexity obtained by fractional centrifugation, centrifugation through sucrose gradients, treatment with a variety of proteolytic enzymes and deproteinization by chemical extractions. The simple haptene of low molecular weight displayed no toxicity, and the purified cell walls which are known to consist chiefly of C-polysaccharide[21] were less toxic than certain of the preparations containing macromolecular complexes of intermediate size.

These observations suggest that the manner in which group A streptococcal cell walls are broken down in the tissues of an infected host could influence the toxic properties of the complexes released and the type of tissue damage produced. If the cell walls were broken down promptly to complexes of low molecular weight they might be excreted without inducing damaging effects, as observed by Schmidt.[19] However, if the breakdown of the cell walls in the tissue was less complete and resulted in the release of large macromolecular complexes of the type found in the crude extracts under study, the C-polysaccharide would be capable of producing chronic intermittent and remittent lesions of the connective tissue. The manner in which a given individual would break

down the cell wall of group A streptococci might be influenced by genetic factors and the nature of the previous contacts with group A streptococci experienced by the individual.

Humphrey and Pagel[22] investigated the tissue response to heat-killed group A streptococci injected intracutaneously. In 6 out of 6 "normal subjects" studied, a minimal inflammatory response was observed, and intact cocci lying free in the perivascular spaces and in macrophages were seen in sections prepared 11 to 15 days after the injections were made. Patients with rheumatic fever, rheumatoid arthritis and post-streptococcal erythema nodosum were tested during the period immediately following acute manifestations of their disease. Twenty-one of 26 patients examined showed "hypersensitive" responses having the characteristics of an Arthus type reaction. In specimens from these individuals, procured 11 to 15 days after injection, no cocci were seen in the lesions. These observations suggest that the individuals who showed a "hypersensitive" response, degraded the heat-killed cocci at least at a different rate if not in a different manner than did the "normal" individuals. These findings support the view that there may be a difference in the manner in which individuals degrade cellular components of group A streptococci; this, in turn, could influence the type of toxic macromolecular components liberated and the type of tissue injury produced.

The duration of the reaction under study is interpreted to mean that the toxic macromolecular complex of carbohydrate remains fixed at or near the site of injection for at least 60 days. This suggests that the location within the tissues where the group A streptococci are broken down would likely influence the distribution of lesions observed. The localization of lesions in fatal cases of *Streptococcus hemolyticus* infections was studied by Mallory and Keefer.[23] Lesions were described in the heart, kidney, liver, spleen, lungs and pancreas. They found suppurative and nonsuppurative lesions; some of these contained organisms but many did not. Their observations are compatible with the concept that tissue injury may continue in this infection after intact bacterial cells are no longer observable in the lesions. They suggested that continued damage was due in part to an antigen-antibody reaction. However, it is possible that part of the damage was due to liberation of macromolecular complexes containing the group-specific C-polysaccharide at the time the cell walls of the organisms were degraded.

These workers also discussed the possible relation of the focal lesions in the heart in the cases of active streptococcal infection to the pathogenesis of the Aschoff body in patients who developed rheumatic fever following a streptococcal infection. They stated: "If continued activity or reinfection occurs, such foci could conceivably result in necrosis of

the newly formed collagen, a reaction to this necrotic collagen in the form of giant cells and thus Aschoff body formation."[23] In light of the data presented in this report, it is suggested that the development of these focal lesions, including the formation of Aschoff bodies, could be the result of gradual liberation of the toxic macromolecular complexes of C-polysaccharide from the cell walls of the group A streptococci as they are digested in the focal areas.

In considering the possible role of this toxic material in the pathogenesis of such diseases as rheumatic fever and other chronic disorders of connective tissue, emphasis is placed on the possible liberation of the macromolecular complex from the streptococcal cell wall at or near the site where the lesions, in which bacteria are not observable, occur. The transport in the blood stream of a molecule of this nature and its subsequent localization at the sites where focal lesions are observed, in such disorders as rheumatic fever and rheumatoid arthritis, seem less likely than the localization of viable streptococci at these sites and subsequent liberation of the toxic macromolecule as the bacterial cells disintegrate under the influence of host resistance mechanisms. This concept is in keeping with the selective localization of streptococci observed by Mallory and Keefer[23] in natural infection, and by Angevine and Rothbard[24] and Cecil, Angevine and Rothbard[25] in experimentally infected rabbits; evidence of continued tissue injury after intact streptococcal cells were no longer observable in the lesions was noted in all of these studies.

Attempts are now being made to develop a method to identify the C-polysaccharide of the group A streptococci in the experimental skin lesions. Such a method, if sensitive enough, would permit one to effectively test the theory that the late nonsuppurative sequelae of group A streptococcal infection are due to the toxic effects of macromolecular complexes of the C-polysaccharide. This substance could thus be sought in the lesions associated with these disorders.

Summary

A single intradermal injection of a toxic cellular component of group A streptococci produced a remittent and intermittent nodular lesion of the dermal connective tissue in rabbits. The component has been identified as a macromolecular complex of the group-specific C-polysaccharide. Acute recurrent lesions were observed to develop as late as 53 days after injection. The histologic features of the reaction are described. The possible role of the toxic substance in the pathogenesis of nonsuppurative sequelae of group A streptococcal infection is discussed on the basis of the observations reported. It is suggested that the liberation

of the carbohydrate of group A streptococcal cell walls, in its toxic physicochemical form, would account for continued tissue damage after intact streptococcal cells could no longer be demonstrated by direct or cultural study of the focal lesions characterizing such disorders as rheumatic fever and rheumatoid arthritis.

References

1. Schwab, J. H. An intracellular hemolysin of group A streptococci. I. Influence of sonic energy and pH on hemolytic potency. *J. Bact.*, 1956, **71**, 94–99.
2. Schwab, J. H. An intracellular hemolysin of group A streptococci. II. Comparative properties of intracellular hemolysin, streptolysin S′ and streptolysin O. *J. Bact.*, 1956, **71**, 100–107.
3. Schwab, J. H. and Cromartie, W. J. Studies on a toxic cellular component of group A streptococci. *J. Bact.*, 1957, **74**, 673–679.
4. Cromartie, W. J., and Schwab, J. H. Chronic remittent dermal lesions in rabbits following a single injection of an extract of group A streptococci. (Abstract) *Fed. Proc.*, 1957, **16**, 355.
5. Cromartie, W. J.; Schwab, J. H., and Craddock, J. G. Effects of streptococcal cellular components on dermal connective tissue: A comparative histological study. (Abstract) Bacteriology Proceedings, 1958, 68.
6. Schwab, J. H.; Cromartie, W. J., and Roberson, B. S. Identification of a toxic cellular component of group A streptococci as a complex of group-specific C polysaccharide and a protein. *J. Exper. Med.*, 1959, **109**, 43–54.
7. Margolis, G., and Pickett, J. P. New applications of the luxol fast blue myelin stain. I. A myelo-angio-cytoarchitectonic method. II. A myelin-neuroglia method. III. A myelin-fat method. IV. A myelin-axis cylinder method. *Lab. Invest.*, 1956, **5**, 459–474.
8. Gomori, G. A rapid one-step trichrome stain. *Am. J. Clin. Path.*, 1950, **20**, 661–664.
9. Movat, H. Z. Demonstration of all connective tissue elements in a single section. Pentachrome stains. *A.M.A. Arch. Path.*, 1955, **60**, 289–295.
10. Mallory, F. B. Pathological Technique. W. B. Saunders Co., Philadelphia, 1938, p. 164.
11. Maximow, A. A. and Bloom, W. A. A Textbook of Histology. W. B. Saunders Co., Philadelphia, 1949, ed. 5, p. 50.
12. Movat, H. Z., and More, R. H. The nature and origin of fibrinoid. *Am. J. Clin. Path.*, 1957, **28**, 331–353.
13. Glynn, L. E., and Loewi, G. Fibrinoid necrosis in rheumatic fever. *J. Path. & Bact.*, 1952, **64**, 329–334.
14. Roberson, B. S.; Schwab, J. H., and Cromartie, W. J. Some physicochemical properties related to toxic activity of a cellular component of group A streptococci. (Abstract) Bacteriology Proceedings, 1959, 67.
15. Felton, L. D. The significance of antigen in animal tissues. *J. Immunol.*, 1949, **61**, 107–117.
16. Schwab, J. H., and Cromartie, W. J. Immunological studies on a C polysaccharide complex of group A streptococci having a direct toxic effect on connective tissue. *J. Exper. Med.*, 1960, **111**, 295–307.

17. Rantz, L. A., and Randall, E. Antibacterial precipitating antibodies in a group A hemolytic streptococcus sore throat. *Am. J. Med.*, 1947, **2**, 551–567.
18. Halbert, S. P.; Swick, L., and Sonn, C. The use of precipitin analysis in agar for the study of human streptococcal infections. I. Oudin technic. II. Ouchterlony and Oakley technics. *J. Exper. Med.*, 1955, **101**, 539–576.
19. Schmidt, W. C. Group A streptococcus polysaccharide: studies on its preparation, chemical composition, and cellular localization after intravenous injection into mice. *J. Exper. Med.*, 1952, **95**, 105–118.
20. Zittle, C. A., and Harris, T. N. The antigenic structure of hemolytic streptococci of Lancefield Group A. X. The purification and certain properties of of the group-specific polysaccharide. *J. Biol. Chem.*, 1942, **142**, 823–833.
21. McCarty, M. The lysis of group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. I. Nature of the cellular substrate attacked by the lytic enzymes. *J. Exper. Med.*, 1952, **96**, 569–580.
22. Humphrey, J. H., and Pagel, W. The tissue response to heat-killed streptococci in the skin of normal subjects, and in persons with rheumatic fever, rheumatoid arthritis; subacute bacterial endocarditis and erythema nodosum. *Brit. J. Exper. Path.*, 1949, **30**, 282–288.
23. Mallory, G. K., and Keefer, C. S. Tissue reactions in fatal cases of streptococcus haemolyticus infection. *Arch. Path.*, 1941, **32**, 334–355.
24. Angevine, D. M., and Rothbard, S. The significance of the synovial villus and the ciliary process as factors in the localization of bacteria in the joints and eye of rabbits. *J. Exper. Med.*, 1940, **71**, 129–135.
25. Cecil, R. L.; Angevine, D. M., and Rothbard, S. Experimental arthritis in rabbits produced with streptococci and other organisms. *Am. J. M. Sc.*, 1939, **198**, 463–475.

[*Illustrations follow*]

Legends for Figures

Except where indicated, photographs were prepared from sections stained with hematoxylin and eosin.

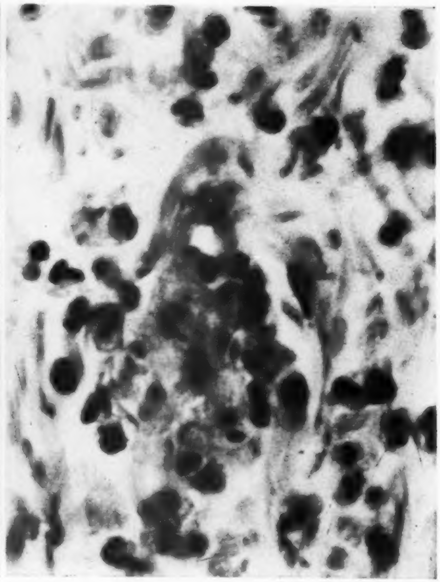
Fig. 1. A group of nodules which appeared on the 17th day after a single intradermal injection of extract of group A streptococci. A similar nodular lesion appeared in the same skin area on the fifth day and completely subsided on the twelfth day after injection. Photographed 18 days after injection.

Fig. 2. A lesion 3 hours after injection of group A streptococcal extract. Focal alteration of the collagen bundles is visible in this early lesion. Deeply stained, rather homogeneous appearing eosinophilic substance is present in the area of alteration. × 600.

Fig. 3. A lesion 9 days after injection of group A streptococcal extract. Thickening of the dermis is associated with an acute and chronic inflammatory reaction. Areas of acute necrosis appear to be coalescing. An acute exacerbation of a subsiding lesion occurred in this animal 8 days after the injection was made, and the lesion was procured on the ninth day. × 20.

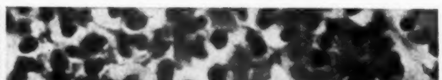
Fig. 4. A lesion during the first day after injection by group A streptococcal extract. The bundles of collagen are replaced by bands of homogeneous eosinophilic material. × 600.

1



2

3



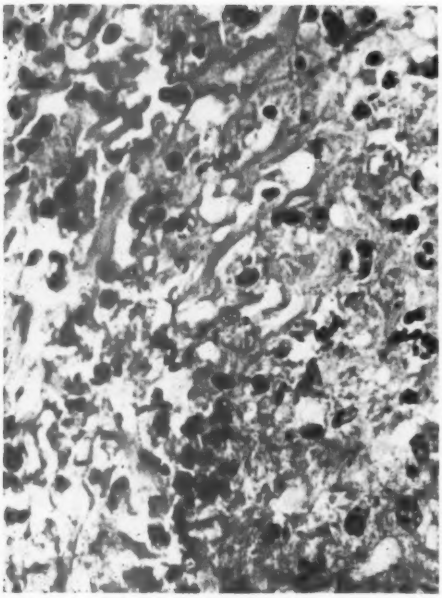
4

FIG. 5. A lesion which appeared during the second day after injection of group A streptococcal extract. Tissue was obtained on the third day. Altered collagen bundles are visible. × 600.

FIG. 6. A lesion which appeared on the fifth day after injection of group A streptococcal extract. Section was prepared on the sixth day after injection. Marked swelling of the collagen bundles is seen. The relatively normal appearing collagen bundles stain blue and appear dark in the photograph. The more markedly altered bundles stain rose to pink and appear less dark than the areas which are stained blue. Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 600.

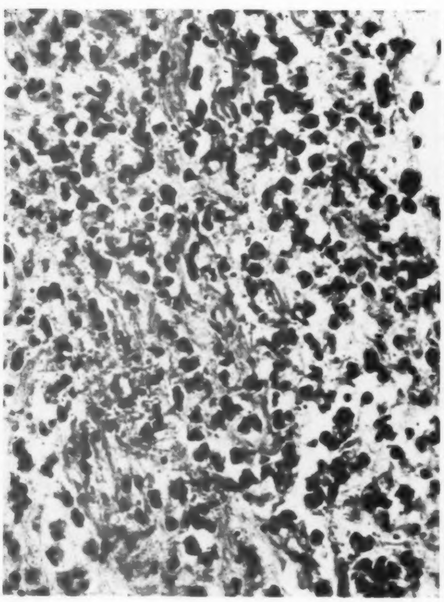
FIG. 7. A lesion 53 days after injection of Group A streptococcal extract. An acute exacerbation of a chronic lesion occurred in this animal 2 days before the specimen was procured. The alteration of the collagen bundles and the infiltration with heterophils is similar to that seen in nodules which developed 3 hours to 1 day after injection of the group A streptococcal extract. × 400.

FIG. 8. A lesion 22 days after a single injection of group A streptococcal extract. An acute exacerbation of a chronic lesion occurred in this animal 2 days before the specimen was obtained. Diffuse infiltration of tissue with mononuclear cells is seen. One giant cell is present. A focus of acute necrosis may be noted in lower right corner of the picture. × 400.

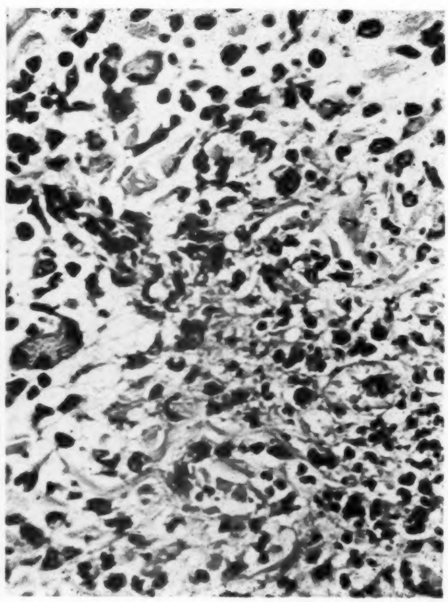


5

6



7



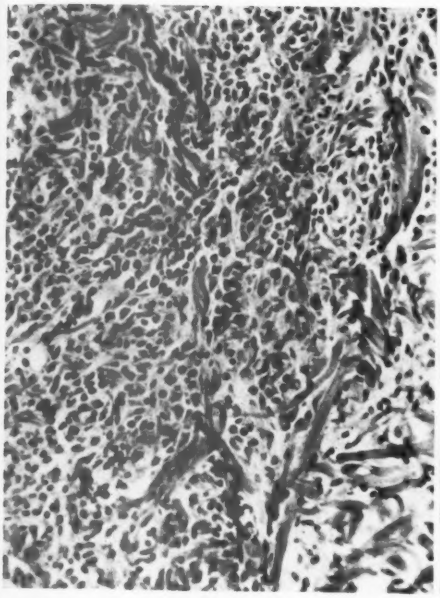
8

FIG. 9. A lesion 31 days after injection of group A streptococcal extract. An acute relapse of a lesion that had appeared to subside completely occurred in this animal 15 days after injection of the extract. This lesion evolved into a firm plaque-like area that was present when the animal was sacrificed on the 31st day after injection. The presence of many macrophages is the most striking feature visible in this section. × 200.

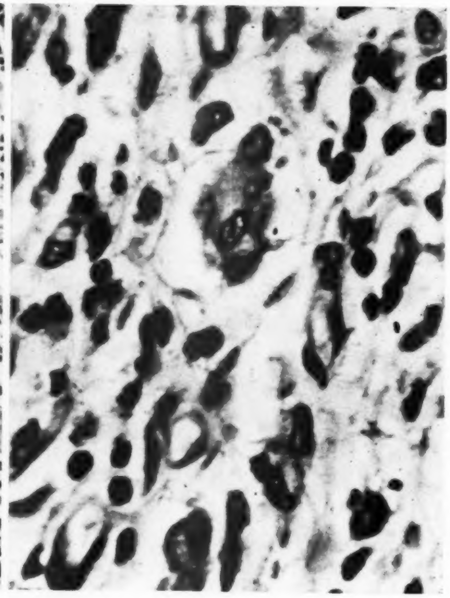
FIG. 10. A lesion from an animal sacrificed 31 days after injection of group A streptococcal extract. A plaque-like nodular lesion had been present in this animal for 12 days before the section was procured. One of the various types of giant cells observed in these lesions is illustrated. × 800.

FIG. 11. The site where group A streptococcal extract was injected 21 days before the animal was sacrificed. An area of necrosis is visible along the right side of the photograph. A chronic reaction with mononuclear cell infiltration is present adjacent to the area of necrosis. The site where extract of *Streptococcus mitis* was injected into this animal, procured at the same time, showed no microscopic change. × 200.

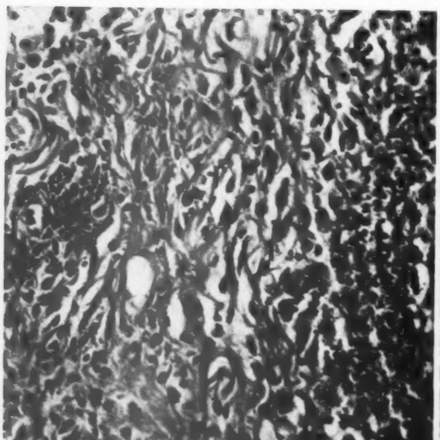
FIG. 12. Twenty-four hours after a second injection of group A streptococcal extract. This animal had received an injection of group A streptococcal extract 60 days before the second injection was made. The lesions resulting from the first injection subsided completely 6 days before the second injection was administered. There is dense infiltration with heterophils and what seem to be a few fragments of collagen bundles. Absence of necrosis and thrombosis of blood vessels may be noted. × 200.



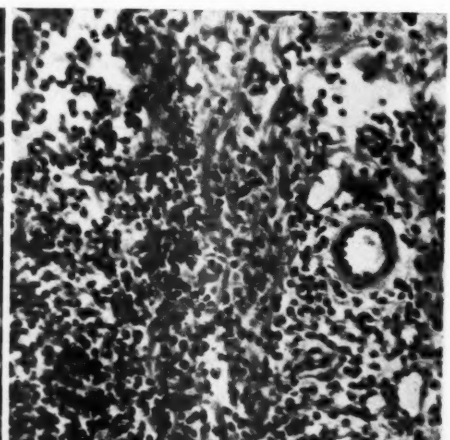
9



10



11



12

A COMPARATIVE MICROMORPHOLOGIC STUDY OF NORMAL HUMAN EPIDERMIS AND A HUMAN SQUAMOUS CELL CARCINOMA TRANSPLANT

George A. Edwards, Ph.D.,* and Laszlo Makk

From the Division of Laboratories and Research, New York State Department of Health, and the Albany Medical College of Union University, Albany, N.Y.

During the past decade electron microscopy has elucidated many of the fine structural details of the nucleus and nuclear-cytoplasmic exchange,[1-3] cytoplasmic organization,[4,5] membrane systems, in particular the endoplasmic reticulum,[6-8] Golgi complex,[9,10] and mitochondria.[10,11] With the exception of the virus tumor, less is known of the fine structure of neoplastic cells and tissues. Few comparative studies of normal and tumorous cells have been presented.[12-14] Recently the fine structure of tumors and their possible viral etiology has been reviewed.[15]

Pure description of abnormal cells does not suffice, however, for the interpretation of the changes from normal, which accompany, cause, or result in neoplasm. It seems logical, therefore, to attempt the investigation of systems which permit of a certain modicum of control, e.g., cell transformation systems[16,17] and tumor transplants.[18,19] With that view in mind, the present investigation has as its aim the comparison of the fine structure of two different stages of a squamous cell carcinoma transplant with normal human infant and adult epidermis, and with previous reports on the fine structure of skin. The results give some clue as to several of the variations from the normal pattern and permit some interpretations of the mechanisms involved in the deviations.

Material and Methods

The control specimens in this study were of normal human squamous epithelium from the foreskin of infants and from the thigh of an adult. The transplants were of early and late 13th generation of the A21 human squamous cell carcinoma of the penis (grade II) cultured in the cheek pouches of cortisone-treated hamsters.[20] Sections were obtained in each of the tumors at various levels from the surface through to the center of the tumor nodule.

All tissues were fixed with 2 per cent osmium tetroxide buffered with

This study was aided by a grant from the American Cancer Society.

Received for publication, October 9, 1959.

* Deceased, March 1, 1960.

veronal acetate to pH 7.4. The tissue was dehydrated in a graded series of ethanols, infiltrated with a butyl-methyl methacrylate mixture in the proportion of 9:1, and embedded in a similar mixture containing 1.5 per cent Lucidol as initiator. Polymerization was carried out at 46° C. for 36 hours, the specimens being subsequently cured for several days at room temperature before sectioning. Sections were cut with a Fernández-Morán diamond knife in a Porter-Blum microtome, mounted on grids covered with formvar stabilized with a thin layer of carbon, and observed in a Siemens' Elmiskop I.

Results

Normal Skin

The layer of normal skin most closely related morphologically to the more normal appearing cells of the carcinoma transplant is the stratum germinativum of the epidermis; hence, our description will be restricted to that layer. The cells are roughly cuboidal in shape (Fig. 1) and equal in size. The intercellular spaces are narrow, giving the tissue a closely packed appearance. Adjacent cells are multiply attached to each other by desmosomes in a manner similar to the meshing of gears (Figs. 1 to 5).

The nucleus (Fig. 1) of the normal epidermal cell is large, generally centrally located, round to elliptical and of smooth contour. The nuclear matrix is granular to filamentous. Chromatin is generally peripheral. The nucleolus is often small and frequently vacuolated (Fig. 1). The nucleus is limited by the nuclear membrane proper, the perinuclear cisterna, and the outer nuclear envelope. The perinuclear cisterna is generally narrow but in some cells appears wide, irregular, and continuous with perinuclear lacunas of the endoplasmic reticulum. The latter is not prominent in the cells of the stratum germinativum, appearing usually in the form of a few scattered macrovesicles (Figs. 1, 2 and 5). The cytoplasmic matrix consists of extremely fine filaments with little electron density. Numerous ribonucleoprotein (RNP) granules are uniformly distributed throughout the cytoplasmic area. The mitochondria (Figs. 1 and 5) are relatively small, spherical to cylindrical, contain a dense matrix and but few cristas. The Golgi complex is usually small, comprises dictyosomal and vesicular elements and is situated close to the nucleus.

The salient feature of these cells is the "cytoplasmic skeleton" of tonofibrils forming a tridimensional framework around the nucleus and radiating out to the cell border. Tonofibrils are composed of irregular bundles of tightly packed tonofilaments. They are most numerous immediately surrounding the nucleus (Figs. 1, 2 and 5). In some sections,

continuity of perinuclear and peripheral filaments is seen (Fig. 5). Indeed, a single bundle of filaments may directly connect perinuclear tonofibrils with one or several intercellular bars (Fig. 3). The peripheral tonofilament bundles may be interconnected (Fig. 2), but in all cases they terminate in the cytoplasmic region of the desmosomes. Interestingly enough, they may terminate either normal to, or parallel with, the long axis of the desmosome (Figs. 3 and 4).

In their morphologic detail, the desmosomes show the same characteristics as those from various other tissues. A desmosome consists of two closely apposed modified plasma membranes. The modification is seen as a series of interruptions of the double-leaved unit membrane, bordered on the cytoplasmic side by parallel rows of filaments, and on the intercellular side by oriented rows of material with somewhat less electron density. The nonattachment regions of the plasma membranes are generally smooth in contour, but occasionally long, often branched, digitations of the border occur (Figs. 2 to 4).

Squamous Cell Carcinoma Transplant

The nodules from both early and late stages contained the same types of cells, hence will be considered as one. The structure of the cells varied from the periphery to the center of a nodule. The surface cells have an appearance more nearly resembling the normal epidermal cells described above. The cells in the interior are greatly changed from the normal. In general, the transplant nodule is featured by loosely applied tumor cells, among which occur capillaries, extravasated blood cells, fibrocytes, collagen, and even nerve.

The first notable difference from normal is the looseness of the tissue (Figs. 6 and 7); i.e., intercellular spaces are wide, variable, and the number of desmosomes greatly diminished, there being usually not more than 3 to 4 connections between cells in a given plane of sectioning. Coincident with the reduction in numbers, there is a change in the microstructure of the desmosomes. They are most generally short and straight (Fig. 6), although in a few instances (Fig. 9) convoluted desmosomes or wisps of intercellular material (Fig. 10) between unmodified plasma membranes occur. A typical desmosome between tumor cells is shown in detail in Figure 11. The cell borders are essentially parallel, there is little dense material in the intercellular space, and there are no tonofilaments associated with the cytoplasmic elements of the desmosome.

Cells from the surface of nodules are shown in Figures 6 to 8. In these the nuclei are spherical (Fig. 6) to highly irregular and large (Fig. 7). The nucleoli are greatly enlarged and vacuolated. The matrix of the nucleus is noticeably denser than normal and contains uniformly dense,

small granules and a few scattered aggregates of larger granules of variable size and density. The number of nuclear pores appears to be increased in the tumor cells as compared with normal cells. Mitochondria are numerous, either scattered throughout the cell (Figs. 6 and 7) or aggregated in clusters in the perinuclear region. A few mitochondria appear cylindrical (Fig. 8), but the vast majority are rounded. The mitochondria possess few cristas, which are often recurrent (Figs. 7 and 8). The matrix is composed of only a few filaments (Fig. 8) giving the mitochondrion a "ghost" appearance.

The Golgi complex (Fig. 8) in the surface cells does not vary too greatly from normal. It is usually situated near, or in, a nuclear pocket, and comprises dictyosomal and vesicular elements. Perhaps it is significant that in the surface cells the microvesicular component predominates. The endoplasmic reticulum is variable in quantity, type, and distribution in the cells at the periphery of the nodule. In general, however, it is more prominent than in normal cells. The reticulum may appear as a few peripheral vesicles of smooth membrane profile. More typically it is seen as long, tortuous, and often branched tubules and lacunas of rough membrane profile (Figs. 6 and 8). Associated with the latter membranes are numerous RNP granules. The RNP granules free in the cytoplasmic matrix are clustered into rosettes, rather than having uniform distribution as single particles, as in the normal. In addition to the RNP granules, the cytoplasmic matrix contains fine filaments (Fig. 8) apparently unrelated to tonofilamentous material.

Few tonofilaments are visible in these cells, generally appearing in the perinuclear cytoplasmic area only (Fig. 6). The tonofilaments are formed into very short bundles with little indication of interconnections. The filaments themselves (Fig. 12) are morphologically similar to those of the normal. However, in some instances (Fig. 13) filaments are seen to be continuous with cytoplasmic inclusions that have the appearance of degenerated tonofibrils. In other instances (Fig. 14) a few patches of filaments occur among the microvesicular elements of the Golgi complex. No tonofilaments occur in the periphery of the cell, nor are tonofilaments associated with desmosomes.

Deeper in the nodules the tumor cells no longer resemble the normal cells in any fashion. Hypertrophy of the membranes of the Golgi complex and of the endoplasmic reticulum is seen in many of the cells at this level, ranging from scattered, isolated membrane areas to complete filling of the cytoplasm (Fig. 15) with reticular and Golgi membranes. The endoplasmic reticulum is of the rough profile type. The Golgi complex consists of horseshoe-shaped arrays of lamellated saccules surrounding microvesicles. The mitochondria in this type of cell are few,

small, rounded, and "empty"; their outer membranes are often incomplete.

Other cells deeper in the nodule present a rather different pattern (Figs. 16 and 17). These cells are filled predominantly with what appear to be modified mitochondria. The recognizable mitochondria possess the usual double limiting membrane, the inner one of which may form a few incomplete cristas (Fig. 16). Several mitochondria may be linked together by their outer membranes and perinuclear mitochondria may be attached to the outer nuclear membrane. Matrices of these mitochondria vary from a few filaments to little or no electron dense substance. In the same cells there are membrane-limited structures of a size and shape which suggest their being modified mitochondria (Fig. 17). Little or no matrix is visible in these structures. Their membrane at times appears double, but in general it is thick and extremely dense. Invaginations of the membrane suggest vestigial cristas.

Cells of the interior, sometimes seen to have incomplete borders, are characterized by whorls of membranes often resembling myelin forms (Figs. 18 to 20). In the simplest form (Fig. 18) the membranes appear as derived from, and forming a few loose turns of, smooth membrane profiles of aggregated lacunas of the endoplasmic reticulum, often enclosing tiny islands of cytoplasmic matrix. In more complex forms (Figs. 19 and 20) the whorls appear as tightly packed membranes. In this form they most closely resemble myelin packing. Cells showing a greater number of interruptions of the cell border usually possess a cytoplasm entirely filled with whorls of various sizes from the small size enclosing cytoplasmic islands up to large compound whorls and vacuoles lined by fragments of tightly packed membranes. The residual cytoplasm (Fig. 20) of such cells consists of few disrupted or modified mitochondria and few vesicles of the smooth membrane profiled endoplasmic reticulum.

Discussion

Significant ultrastructural differences were not observed between the strata germinativa of the infantile foreskin and the adult epidermis; hence they may be considered together as normal human squamous epithelium. Further, in most respects the results of the present investigation of normal skin were confirmatory of previous studies by others.[21–28]

The first major difference between the tumor transplant and the normal skin is seen in the loose packing of the tumor cells. This phenomenon may be correlated with several factors. It is a general characteristic of tumor cells and cells in tissue culture to lack a basement membrane, thus reducing the possibilities for intercellular cohesion.

Further, a morphologic characteristic of all tumor cells is the loss of, or lack of, polarity. From the present investigation it appears that the looseness of the tissue is related to the decrease and modification of both tonofibrils and desmosomes. In support of this assumption is the finding by Coman [29] that the adhesive forces between two malignant squamous carcinoma cells are approximately one third of those between two normal squamous epithelial cells.

The desmosomes (i.e., terminal bars, intercellular bars, intercellular bridges, prickles, and nodules of Bizzozero) observed in the normal skin were similar to those previously described.[30] A few details not covered by other reports are perhaps worthy of mention. The plasma membranes of the desmosome are not thicker than the contiguous membranes. Rather, the double-leaved unit membranes are discontinuous or vesiculated. The apparent increased thickness of the membranes observed by others is explained by the accumulation of microfilaments on the cytoplasmic side of the plasma membrane. The intercellular substance usually appears to be longitudinally oriented. However, in some cases transversely oriented arrangements are visible, suggesting interaction of molecules of the adjacent cells. The differences between the desmosomes of the tumor cells and the normal skin are essentially a lack of tonofilaments and a reduction in the intercellular material. The desmosomes normally play a dual role. They appear to be ubiquitous and one of the most important methods of cell contact.[31] They also serve as orientation and anchor points for the tonofilaments.[25] The present study confirms the statement of Selby [21,22] that filaments do not pass from one cell to the other across the intercellular space of the desmosome. The oriented intercellular material observed in the present study is probably a mucoprotein [32] and is the intercellular cement, the loss of which would, in great part, account for the looseness of the tissue and perhaps, as previously suggested,[16] play a role in the process of metastasis.

The nuclei of tumor cells show great variation. In the squamous cell carcinoma transplant the nuclei were larger and more irregular than those of normal epidermis. Furthermore, the nuclear matrix contained some large, dense granules not observed in the normal nuclei. The most striking nuclear variation was the hypertrophy and vacuolation of the nucleolus. In this respect, the carcinoma cells are similar to those of the Rous sarcoma, the endothelioma of Murray-Begg [33,34] and to a bronchogenic carcinoma transplant.[18,19]

Most squamous cell tumors in advanced stages have few or no tonofibrils [35,36] and little or no keratin.[37] It is noteworthy that no keratin layers were seen in the squamous carcinoma transplants presently under investigation. Further, tonofibrils were few or absent in these cells, and

keratohyaline granules were not observed. The two phenomena are probably closely related in view of previous studies of keratin formation. The earlier literature is well summarized by Montagna.[38] Keratin has been thought to originate from keratohyaline which in turn was considered to be formed from either epidermal fibrils, basophilic ground cytoplasm, nucleus, nucleolar extrusions, cell membrane, Golgi apparatus or metamorphosed mitochondria. The most recent hypothesis,[24] quite well documented, suggests that keratin is formed from both tonofilaments and keratohyaline granule substance. The reduction in tonofilaments and the lack of keratohyaline granules and identifiable keratin in the tumor transplant would confirm Brody's thesis. However, membranous or filamentous structures do occur in tumor cells, and these may in fact represent an incomplete process in the formation of the ground filaments around which keratin would normally form.

There are several possible explanations of the diminution of tonofibrils in the tumor cells. Tonofilaments could be produced normally, and degenerate secondarily. However, there were few, and highly localized, filaments in the cells at the surface of the tumor, and only in a few instances did there appear to be degenerated bundles of filaments. It seems unlikely that a normal process of tonofilament formation could exist in these cells. The most fitting possibility is that of an altered nucleocytoplasmic exchange resulting in the reduction or loss of the ability of the cell to form tonofilaments. This idea is based on the fact that normally the tonofilaments are formed immediately outside the nucleus and are often related to nuclear pores, and that in the tumor cells only incomplete perinuclear filaments may be present.

Tumor mitochondria are characteristically few in number,[13] and "empty," i.e., they possess few and incomplete cristas and a matrix of little density.[15] The mitochondria of the squamous cell carcinoma transplant proved no exception to this general rule. They were small, few, and "empty." However, an increase in number was observed in those cells showing hypertrophy of the endomembrane systems. An unusual mitochondrial development was that in which the membranes became dense and thickened. No explanation is readily available for this phenomenon. It is possible, inasmuch as there is strong evidence of the involvement of mitochondria in the formation of keratohyaline granules, that the mitochondria so observed represent an accumulation of the products of an incomplete or malfunctioning hyaline-producing mechanism.

Several differences exist between the cytoplasmic matrices of the normal and the squamous carcinoma cells. The most noticeable are: the appearance of fine filaments in the general ground substance of the tumor cell; the clustering of RNP granules in the cytoplasm; and finally

the aggregation of the granules to the membranes of the endoplasmic reticulum in the tumor cells. The significance of these differences is unknown. It can only be presumed that they are related to the increased protein synthesis involved in the replication or synthesis *de novo* of endomembranes in the late stages of the tumor cell cycle.

Characteristically, the cells in the interior of the nodule showed an almost wild hypertrophy of membranes of the Golgi complex and ergastoplasm. Such endomembrane hypertrophy has also been seen in other cells which are or may become malignant.[16,18,19] This raises several interesting questions. The increased ergastoplasm can be related to increased protein synthesis.[6] The hypertrophy of the Golgi complex is most logically related to increased excretory and feedback activity, in that the Golgi complex appears involved in the extrusion of particles from infected cells.[15,39] It is increased in amount and associated with multivesicular bodies in active cells[17] whether normal or infected. The involvement of the Golgi complex in the removal of waste metabolic products or particulate material is rather readily understood. Indeed, it seems likely, in general, that the microvesicles of the Golgi complex play the same role in active transport out of the cell that is played by the pinocytotic vesicles in active transport into the cell. In the role of a feedback mechanism for the reclamation, storage, and re-utilization of nuclear and cytoplasmic products of low molecular weight, it is perhaps significant that hypertrophy of the Golgi complex occurs during a change-over of cell function, e.g., from rest to active secretion in normal parietal cells or from keratin formation to whorl formation in the tumor cells. The question still remains whether the endomembrane hypertrophy represents a turnover of lipoproteins within the cell, a synthesis *de novo* from nonmembrane material, or a replication of membranes. Although absolute proof is lacking, it seems likely, on the basis of the quantity of membrane formation in trypsinized cells[16] and in the tumor cells presently investigated, that synthesis of lipoprotein membranes is occurring *de novo* and most likely involving RNP granules.

SUMMARY

A human squamous cell carcinoma transplanted to cortisone-treated hamsters has been compared by electron microscopy with normal human squamous epithelium.

The strata germinativa of infantile foreskin and adult thigh epidermis were alike in their morphologic characteristics, and similar to that previously described in other electron microscopic studies. For comparative purposes, however, ultrastructural details are given of some of the cells and the intercellular relations.

The types of cells of the small nodules of both early and late 13th generation carcinoma transplants were the same. However, within a given nodule the cells differed from surface to center. Wide, intercellular spaces occurred among the tumor cells, coincident with a reduction in numbers of desmosomes, and with smooth outline or lack of digitation of the plasma membrane. Cells from the surface of the nodule showed few and modified desmosomes, few tonofilaments, enlarged nuclei, enlarged and vacuolated nucleoli, empty mitochondria, RNP granules in rosettes, small Golgi complexes and little endoplasmic reticulum.

Cells deeper within the nodule possessed hypertrophied endoplasmic reticulum and Golgi apparatuses, lacked tonofilaments, showed incomplete and empty mitochondria, had an increased number of nuclear pores but few or no desmosomes. The cytoplasm of deeper cells in the center of the tumor nodules (presumably the more anaplastic) was filled with whorls of membranes and modified, densely outlined mitochondria, thus showing the most marked changes from the structure of the normal epithelium.

References

1. Watson, M. L. The nuclear envelope. Its structure and relation to cytoplasmic membranes. *J. Biophys. & Biochem. Cytol.*, 1955, **1**, 257–270.
2. Anderson, E., and Beams, H. W. Evidence from electron micrographs for the passage of material through pores of the nuclear membrane. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, No. 4 Suppl., 439–444.
3. Bernhard, W. Ultrastructural aspects of nucleo-cytoplasmic relationship. *Exper. Cell Res.*, 1959, **6**, Suppl., 17–50.
4. Porter, K. R., and Kallman, F. L. Significance of cell particulates as seen by electron microscopy. *Ann. New York Acad. Sc.*, 1952, **54**, 882–891.
5. Porter, K. R. The Submicroscopic Morphology of Protoplasm. In: The Harvey Lectures, 1955–1956. Academic Press, Inc., New York, Series **51**, 1957, pp. 175–228.
6. Porter, K. R. Electron microscopy of basophilic components of cytoplasm. *J. Histochem.*, 1954, **2**, 346–375.
7. Palade, G. E. The endoplasmic reticulum. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, No. 4 Suppl., 85–98.
8. Haguenau, F. The Ergastoplasm: Its History, Ultrastructure, and Biochemistry. In: International Review of Cytology. Bourne, G. H., and Danielli, J. F. (eds.). Academic Press, New York, 1958, **7**, 425–483.
9. Dalton, A. J., and Felix, M. D. Cytologic and cytochemical characteristics of the Golgi substance of epithelial cells of the epididymis—in situ, in homogenates, and after isolation. *Am. J. Anat.*, 1954, **94**, 171–207.
10. Dalton, A. J., and Felix, M. D. Electron Microscopy of Mitochondria and the Golgi Complex. Symposia of the Society for Experimental Biology, Academic Press, New York, 1957, **10**, Mitochondria and Other Cytoplasmic Inclusions, 148–159.
11. Siekevitz, P. Oxidative phosphorylation in muscle mitochondria and its possible regulation. *Ann. New York Acad. Sc.*, 1959, **72**, 500–514.

12. Dalton, A. J., and Felix, M. D. The electron miscroscopy of normal and malignant cells. *Ann. New York Acad. Sc.*, 1956, **63**, 1117–1140.

13. Howatson, A. F., and Ham, A. W. The Fine Structure of Normal and Malignant Cells as Revealed by the Electron Microscope. Proceedings of the Second Canadian Cancer Research Conference, 1956. Academic Press, 1957, 17–58.

14. Howatson, A. F., and Ham, A. W. The fine structure of cells. *Canad. J. Biochem. & Physiol.*, 1957, **35**, 549–564.

15. Bernhard, W. Electron microscopy of tumor cells and tumor viruses. A review. *Cancer Res.*, 1958, **18**, 491–509.

16. Edwards, G. A., and Fogh, J. Micromorphologic changes in human amnion cells during trypsinization. *Cancer Res.*, 1959, **19**, 608–611.

17. Fogh, J. and Edwards, G. A. Ultrastructure of primary culture amnion cells and transformed FL cells in continuous culture. *J. Nat. Cancer Inst.*, 1959, **23**, 893–923.

18. Edwards, G. A.; Ruska, C.; Ruska, H., and Skiff, J. V., Jr. Comparison of the Fine Structure of Two Human Carcinomas. Proceedings, Fourth International Congress on Electron Microscopy, Berlin, 1958. Springer Verlag, Berlin, 1960, Vol. 2, pp. 466–470.

19. Edwards, G. A.; Ruska, C.; Ruska, H., and Skiff, J. V., Jr. The micromorphology of a human bronchogenic carcinoma. *Cancer*, 1959, **12**, 982–1002.

20. Skiff, J. V., Jr.; Stein, A. A.; Maisel, M.; Heilbrunn, C., and Hertz, D. Observations on the heterologous transplantation of human tumors. *Cancer Res.*, 1958, **18**, 485–487.

21. Selby, C. C. An electron miscroscope study of the epidermis of mammalian skin in thin sections. I. Dermo-epidermal junction and basal cell layer. *J. Biophys. & Biochem. Cytol.*, 1955, **1**, 429–444.

22. Selby, C. C. An electron microscope study of thin sections of human skin. II. Superficial layers of footpad epidermis. *J. Invest. Dermat.*, 1957, **29**, 131–149.

23. Clark, W. H., Jr., and Hibbs, R. G. Electron microscope studies of the human epidermis: The clear cell of Masson (dendritic cell or melanocyte). *J. Biophys. & Biochem. Cytol.*, 1958, **4**, 679–684.

24. Brody, I. The keratinization of epidermal cells of normal guinea pig skin as revealed by electron microscopy. *J. Ultrastructure Res.*, 1959, **2**, 482–511.

25. Charles, A., and Smiddy, F. G. The tonofibrils of the human epidermis. *J. Invest. Dermat.*, 1957, **29**, 327–338.

26. Charles, A. An electron microscope study of cornification in the human skin. *J. Invest. Dermat.*, 1959, **33**, 65–74.

27. Hibbs, R. G., and Clark, W. H., Jr. Electron microscope studies of the human epidermis. The cell boundaries and topography of the stratum Malpighii. *J. Biophys. & Biochem. Cytol.*, 1959, **6**, 71–76.

28. Odland, G. F. The fine structure of the interrelationship of cells in the human epidermis. *J. Biophys. & Biochem. Cytol.*, 1958, **4**, 529–538.

29. Coman, D. R. Decreased mutual adhesiveness, a property of cells from squamous cell carcinomas. *Cancer Res.*, 1944, **4**, 625–629.

30. Fawcett, D. W. Structural Specializations of the Cell Surface. In: Frontiers in Cytology. Palay, S. L. (ed.). Yale University Press, New Haven, 1958, pp. 19–41.

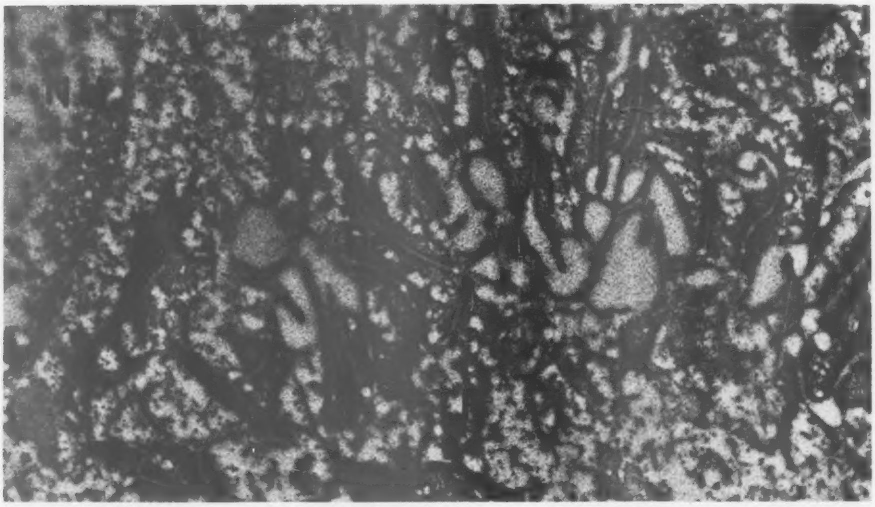
31. WEISS, P. Cell Contact. In: International Review of Cytology. Bourne, G. H., and Danielli, J. F. (eds.). Academic Press, New York, 1958, 7, 391–423.
32. RINALDINI, L. M. J. The Isolation of Living Cells from Animal Tissues. In: International Review of Cytology. Bourne, G. H., and Danielli, J. F. (eds.). Academic Press, New York, 1958, 7, 587–647.
33. BERNHARD, W.; OBERLING, C., and VIGIER, P. L'ultrastructure de virus dans le sarcome de Rous; leur rapport avec le cytoplasme des cellules tumorales. *Bull. Assoc. franç. étude cancer,* 1956, **43,** 407–422.
34. ROUILLER, C.; HAGUENAU, F.; GOLDE, A., and LACOUR, F. L'ultrastructure de l'endothéliome de Murray-Begg. Le problème de l'identification de son agent causal. *Bull. Assoc. franç. étude cancer,* 1956, **43,** 10–22.
35. CONWAY, H. Tumors of the Skin. Charles C Thomas, Springfield, Ill., 1956, 267 pp.
36. LUND, H. Z. Tumors of the Skin. Atlas of Tumor Pathology, Section I, Fascicle 2. Subcommittee on Oncology of the Committee on Pathology of the National Research Council, Armed Forces Institute of Pathology, Washington, D.C., 1957.
37. PILLSBURY, D. M.; SHELLEY, W. B., and KLIGMAN, A. M. Dermatology. W. B. Saunders Co., Philadelphia, 1956, 1331 pp.
38. MONTAGNA, W. The Structure and Function of Skin. Academic Press, New York, 1956, 356 pp.
39. HOWATSON, A. F., and MCCULLOCH, E. A. Virus-like bodies in a transplantable mouse plasma cell tumour. (Letter to the editor.) *Nature, London,* 1958, 181, 1213–1214.

[*Illustrations follow*]

Legends for Figures

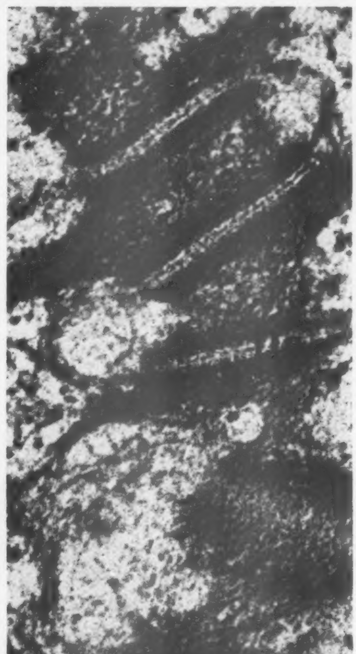
FIG. 1. Normal skin. The stratum germinativum consists of closely packed, cuboidal cells abutting upon numerous, complex desmosomes (De). An oval nucleus (NU) possesses a nucleolus (NCL). Tonofibrils (TF) are seen in various sections throughout the cytoplasm. × 15,000.

1



2

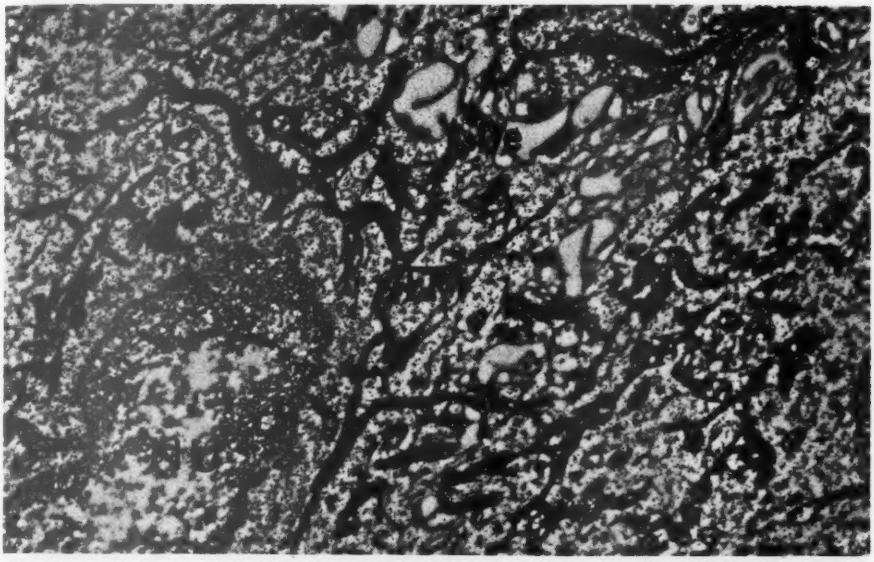
3



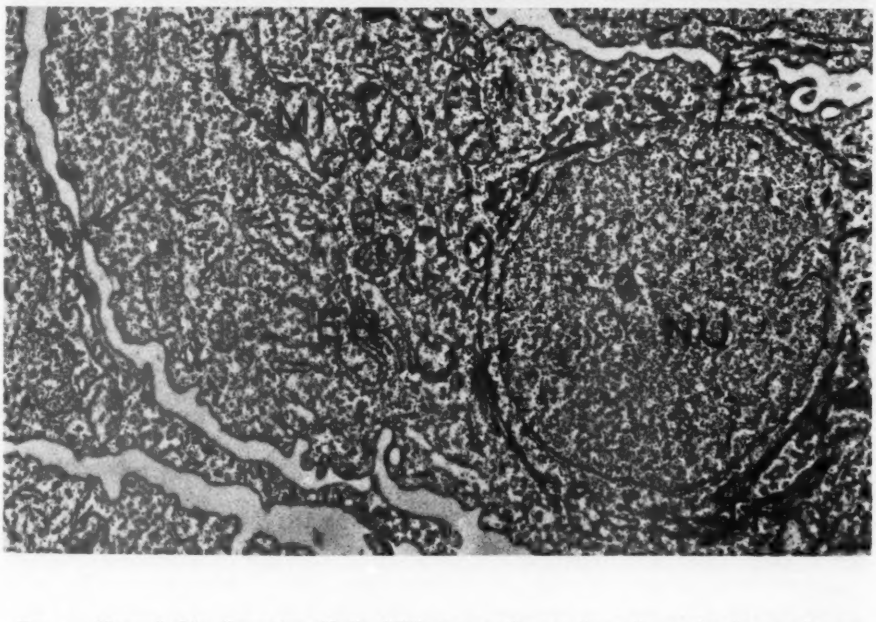
4

FIG. 2. Normal skin. Detail of the abutment of 3 cells by numerous, complex desmosomes (De) continuous with peripheral tonofibrils (TF). × 30,000.

FIGS. 3 and 4. Normal skin. Abutting cells have short, straight desmosomes (De); dense material in the intercellular spaces is probably mucoprotein. Some tonofibrils (TF) connect with desmosomes (compare with Figs. 9 to 11). × 100,000.



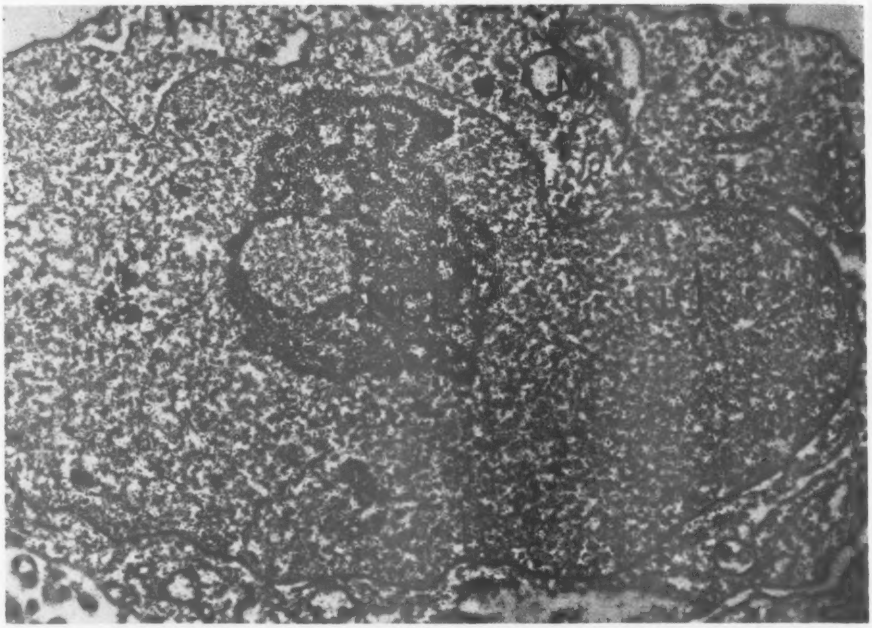
5

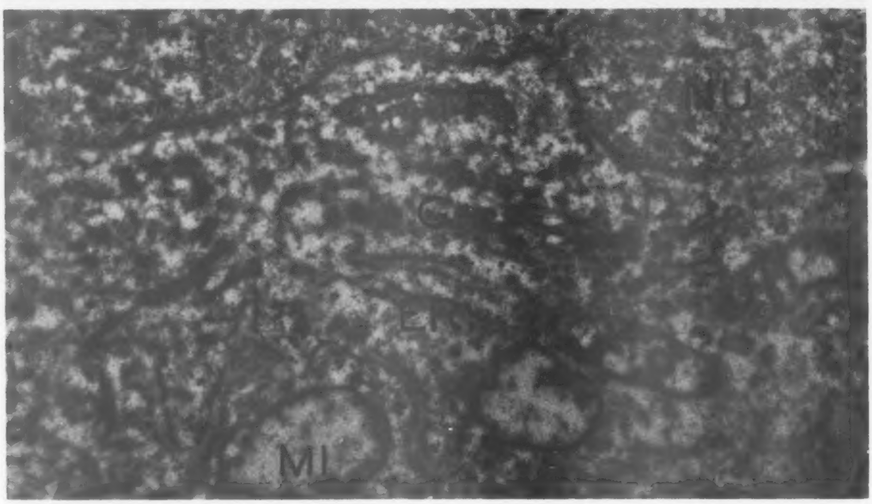


6

FIG. 5. Normal skin. Some tonofibrils (TF) are continuous from the perinuclear region to the desmosomes (De) at the periphery. × 15,000.

FIG. 6. Tumor transplant; loosely packed cells from the surface of the nodule. A few tonofilaments (TF) exist in the perinuclear cytoplasm. Mitochondria (MI) are relatively empty; endoplasmic reticulum (ER) is of the rough membranous profile type. Intercellular contacts (arrows) are scarce. × 15,000.


7

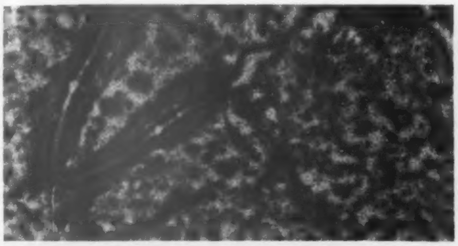


8

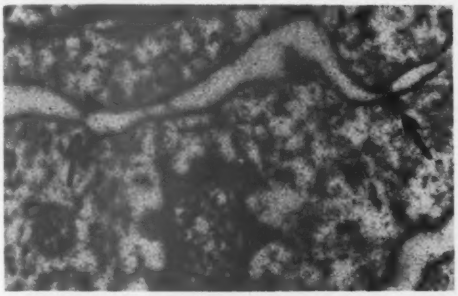
FIG. 7. A cell at periphery of a tumor transplant. Nucleus (NU) and nucleolus (NCL) are swollen; the latter is quite vacuolated. The mitochondria (MI) are essentially "ghost" forms. × 15,000.

FIG. 8. Perinuclear cytoplasm of a tumor transplant cell near the periphery, showing small Golgi complex (G), empty mitochondria (MI), tubules of ergastoplasm (ER) and vestiges of tonofilaments (TF). Cytoplasmic matrix is finely filamentous; the RNP granules are clustered into rosettes. × 30,000.

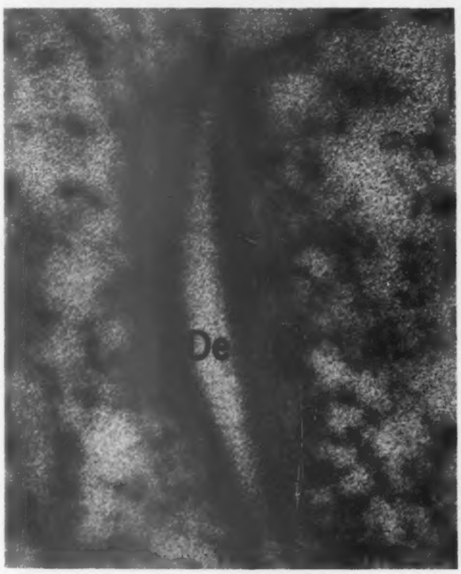
9



10

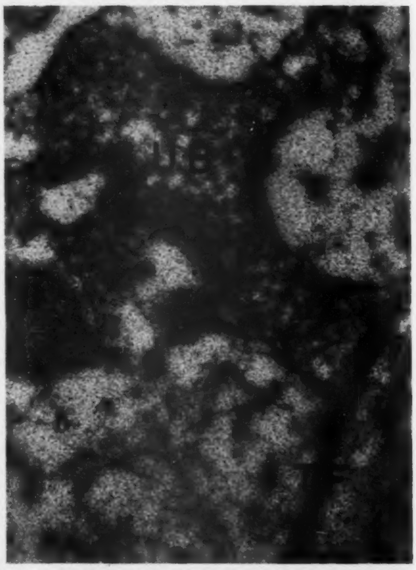


11



12

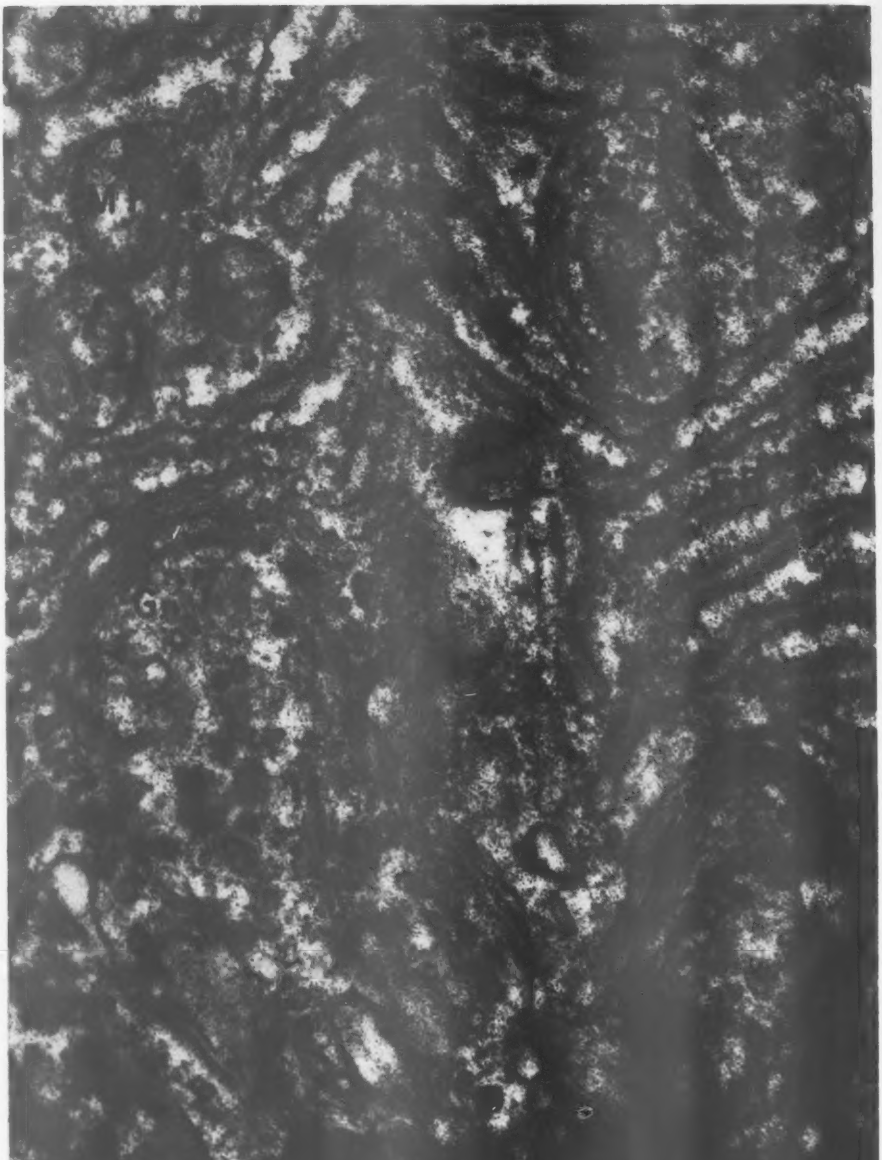
13



14

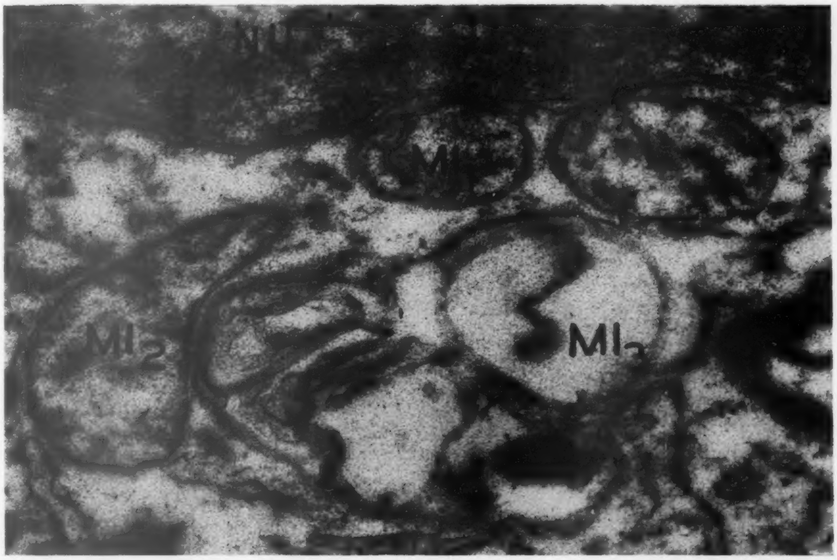
FIGS. 9 to 11. Desmosomes (De) of peripheral cells in tumor nodules (compare with Figs. 2 to 4). The convoluted desmosome (Fig. 9) is the exception. More often they are short modifications of the plasma membrane but lack the tonofilamentous component (Figs. 9 and 10). Figs 9 and 10, × 30,000. Fig. 11, × 120,000.

FIGS. 12 to 14. Tonofilaments (TF) from cells near periphery of a tumor transplant. They may appear normal (Fig. 12), or as vestiges related to what may be a degenerating tonofibril (UB) seen here in cross section (Fig. 13) or as scattered, short vestiges of, or incompletely formed, tonofibrils (Fig. 14). × 60,000.

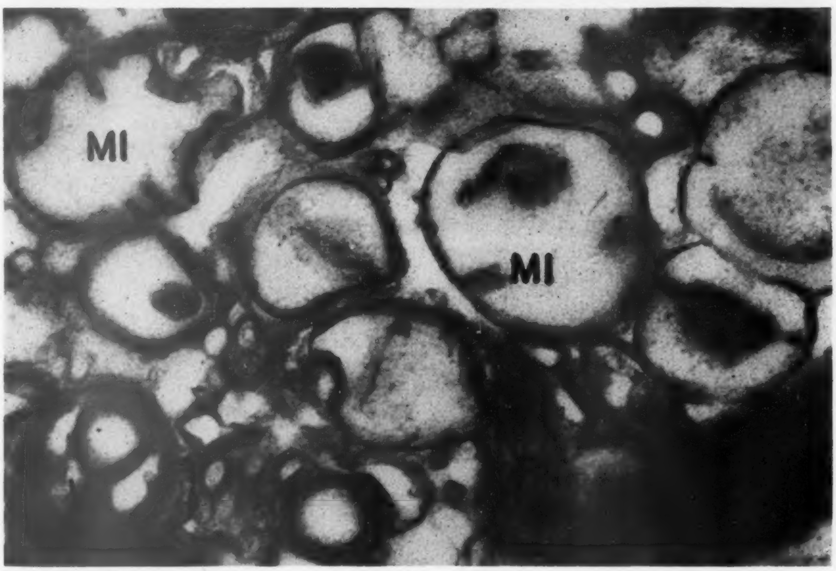


15

FIG. 15. Cytoplasm of a tumor transplant cell near the center of the nodule, showing hypertrophied ergastoplasm (ER) and a Golgi complex (G). Small, empty mitochondria (MI) are seen at the upper left. × 60,000.



16

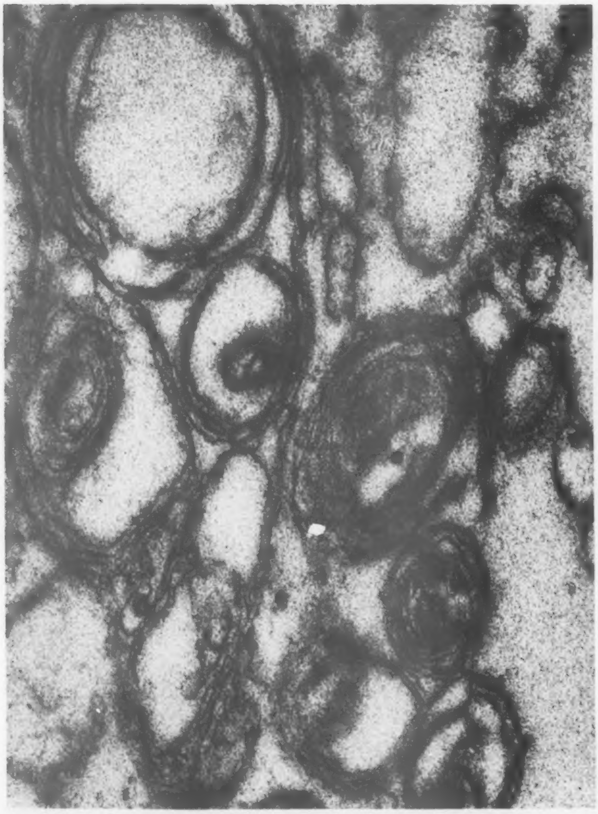


17

FIG. 16. Cell from the center of a transplant nodule. The outer membrane of one mitochondrion (MI_1) is continuous with the outer nuclear (NU) membrane; another (MI_2) has a matrix but incomplete cristas; a third (MI_3) is irregular and empty. × 60,000.

FIG. 17. Apparent modified mitochondria (MI) in a cell from the center of a tumor transplant nodule. These forms are common in cells forming a pearl. Whorls (W) of endoplasmic reticulum and filaments (F) are also common. × 60,000.

18



19

20

FIG. 18. A cell in center of a nodule. Whorls of loosely packed membranes are derived from the endoplasmic reticulum. × 80,000.

FIG. 19. Whorls are tightly packed membranes of endoplasmic reticulum, the packing being closer in the largest whorls. × 70,000.

FIG. 20. Tightly packed membranes, resembling myelin forms. Cell from center of a nodule. × 30,000.